This application is a continuation of U.S. Patent Application Serial No. 09/845,667, filed on April 30, 2001, which is a continuation of U.S. Patent Application Serial No. 09/091,763, filed June 19, 1998, which was a 371 of PCT/GB96/03186, filed December 20,1996

1   Control of protein synthesis, and screening method for
2   agents.
3
4   The present invention relates to the control of
5   glycogen metabolism and protein synthesis, in
6   particular through the use of insulin.
7
8   Many people with diabetes have normal levels of insulin
9   in their blood, but the insulin fails to stimulate
10   muscle cells and fat cells in the normal way (type II
11   diabetes). Currently it is believed that there is a
12   breakdown in the mechanism through which insulin
13   signals to the muscle and fat cells.
14
15   The enzyme glycogen synthase kinase-3 (GSK3) (Embi et
16   al., 1980) is implicated in the regulation of several
17   physiological processes, including the control of
18   glycogen (Parker et al., 1983) and protein (Welsh et
19   al., 1993) synthesis by insulin, modulation of the
20   transcription factors AP-1 and CREB (Nikolaki et al, de
21   Groot et al., 1993 and Fiol et al 1994), the
22   specification of cell fate in *Drosophila* (Siegfied et
23   al., 1992) and dorsoventral patterning in *Xenopus*
24   embryos (He et al., 1995). GSK3 is inhibited by serine

1   phosphorylation in response to insulin or growth
2   factors (Welsh et al., 1993, Hughes et al., 1994, Cross
3   et al., 1994 and Saito et al., 1994) and *in vitro* by
4   either MAP kinase-activated protein (MAPKAP) kinase-1
5   (also known as p90^{rsk}) or P70 ribosomal S6 kinase (p70^{S6k})
6   (Sutherland et al., 1993 and Sutherland et al., 1994).
7
8   We have now found, however, that agents which prevent
9   the activation of both MAPKAP kinase-1 and p70^{S6k} by
10 insulin *in vivo* do not block the phosphorylation and
11 inhibition of GSK3. Another insulin-stimulated protein
12 kinase inactivates GSK3 under these conditions, and we
13 demonstrate that it is the product of the proto-
14 oncogene Akt (also known as RAC or PKB; herein referred
15 to as "PKB").
16
17 GSK3 is inhibited in response to insulin with a half
18 time of two min, slightly slower than the half time for
19 activation of PKBα (one min). Inhibition of GSK3 by
20 insulin results in its phosphorylation at the same
21 serine residue (serine 21) which is targeted by PKBα in
22 vitro. Like the activation of PKBα, the inhibition of
23 GSK3 by insulin is prevented by phosphatidyl inositol
24 (PI-3) kinase inhibitors wortmannin and LY 294002. The
25 inhibition of GSK3 is likely to contribute to the
26 increase in the rate of glycogen synthesis (Cross et
27 al., 1994) and translation of certain mRNAs by insulin
28 (Welsh et al., 1994).
29
30 Two isoforms of PKB, termed PKBα (Coffer & Woodgett,
31 1991), PKBβ (Cheng et al., 1992) and PKBγ (Konishi et
32 al., 1995) have been identified and characterised.
33 PKBβ, also known as RACβ and Akt-2, is over-expressed
34 in a significant number of ovarian (Cheng et al., 1992)
35 and pancreatic (Cheng et al., 1996) cancers and is
36 over-expressed in the breast cancer epithelial cell

1   line MCF7. PKB is composed of an N-terminal pleckstrin

2   homology (PH) domain, followed by a catalytic domain

3   and a short C-terminal tail. The catalytic domain is

4   most similar to cyclic AMP-dependent protein kinase

5   (PKA, 65% similarity) and to protein kinase C (PKC, 75%

6   similarity) findings that gave rise to two of its

7   names, namely PKB (i.e. between PKA and PKC) and RAC

8   (Related to A and C kinase).

9

10   Many growth factors trigger the activation of

11   phosphatidylinositol (PI) 3-kinase, the enzyme which

12   converts PI 4,5 bisphosphate (PIP2) to the putative

13   second messenger PI 3,4,5 trisphosphate (PIP3), and PKB

14   lies downstream of PI 3-kinase (Franke et al., 1995).

15   PKBα is converted from an inactive to an active form

16   with a half time of about one minute when cells are

17   stimulated with PDGF (Franke et al., 1995), EGF or

18   basic FGF (Burgering & Coffer, 1995) or insulin (Cross

19   et al., 1995 and Kohn et al., 1995) or perpervanadate

20   (Andjelkovic et al., 1996). Activation of PKB by

21   insulin or growth factors is prevented if the cells are

22   preincubated with inhibitors of PI 3-kinase (wortmannin

23   or LY 294002) or by overexpression of a dominant

24   negative mutant of PI 3-kinase (Burgering & Coffer

25   1995). Mutation of the tyrosine residues in the PDGF

26   receptor that when phosphorylated bind to PI 3-kinase

27   also prevent the activation of PKBα (Burgering &

28   Coffer, 1995 and Franke et al., 1995).

29

30   The present invention thus provides the use of PKB, its

31   analogues, isoforms, inhibitors, activators and/or the

32   functional equivalents thereof to regulate glycogen

33   metabolism and/or protein synthesis, in particular in

34   disease states where glycogen metabolism and/or protein

35   synthesis exhibits abnormality, for example in the

36   treatment of type II diabetes; also in the treatment of

1   cancer, such as ovarian, breast and pancreatic cancer.
2   A composition comprising such agents is also covered by
3   the present invention, and the use of such a
4   composition for treatment of disease states where
5   glycogen metabolism and/or protein synthesis exhibit
6   abnormality.
7
8   The present invention also provides a novel peptide
9   comprising the amino acid sequence Arg-Xaa-Arg-Yaa-Zaa-
10   Ser/Thr-Hyd, where Xaa is any amino acid, Yaa and Zaa
11   are any amino acid (preferably not glycine), and Hyd is
12   a large hydrophobic residue such as Phe or Leu, or a
13   functional equivalent thereof. Represented in single
14   letter code, a suitable peptide would be RXRX'X'S/TF/L,
15   where X' can be any amino acid, but is preferably not
16   glycine; glycine can in fact be used, but other amino
17   acids are preferred. Typical peptides include
18   GRPRTSSFAEG, RPRAATC or functional equivalents thereof.
19   The peptide is a substrate for measuring PKB activity.
20
21   The invention also provides a method for screening for
22   substances which inhibit the activation of PKB in vivo
23   by preventing its interaction with PIP3 or PI3,4-bisP.
24
25   Thus the invention also provides a method of
26   determining the ability of a substance to affect the
27   activity or activation of PKB, the method comprising
28   exposing the substance to PKB and phosphatidyl inositol
29   polyphosphate (ie PIP3, PI3,4-bisP etc) and determining
30   the interaction between PKB and the phosphatidyl
31   inositol polyphosphate. The interaction between PKB
32   and the phosphatidyl inositol polyphosphate can
33   conveniently be measured by assessing the
34   phosphorylation state of PKB (preferably at T308 and/or
35   S473), eg by measuring transfer of radiolabelled ^{32}P
36   from the PIP3 (for example) to the PKB and/or by SDS-

1   PAGE.

2

3   The method of the invention can also be used for

4   identifying activators or inhibitors of GSK3, such a

5   method can comprise exposing the substance to be tested

6   to GSK3, and (optionally) a source of phosphorylation,

7   and determining the state of activation of GSK3

8   (optionally by determining the state of its

9   phosphorylation.   This aspect of the invention can be

10   useful for determining the suitability of a test

11   substance for use in combatting diabetes, cancer, or

12   any disorder which involves irregularity of protein

13   synthesis or glycogen metabolism.

14

15   The invention also provides a method for screening for

16   inhibitors or activators of enzymes that catalyse the

17   phosphorylation of PKB, the method comprising exposing

18   the substance to be tested to

19   - one or more enzymes upstream of PKB;

20   - PKB; and (optionally)

21   - nucleoside triphosphate

22   and determining whether (and optionally to what extent)

23   the PKB has been phosphorylated on T308 and/or S473.

24

25   Also provided is a method of identifying agents able to

26   influence the activity of GSK3, said method comprising:

27

28   a.   exposing a test substance to a substrate of GSK3;

29

30   b.   detecting whether (and, optionally, to what

31   extent) said peptide has been phosphorylated.

32

33   The test substance may be an analogue, isoform,

34   inhibitor, or activator of PKB, and the above method

35   may be modified to identify those agents which

36   stimulate or inhibit PKB itself.   Thus such a method

1   may comprise the following steps:

2

3   a.   exposing the test substance to a sample containing

4   PKB, to form a mixture;

5

6   b.   exposing said mixture to a peptide comprising the

7   amino acid sequence defined above or a functional

8   equivalent thereof (usually in the presence of Mg^{2+}

9   and ATP); and

10

11   c.   detecting whether (and, optionally, to what

12   extent) said peptide has been phosphorylated.

13

14   In this aspect the method of the invention can be used

15   to determine whether the substance being tested acts on

16   PKB or directly on GSK3. This can be done by comparing

17   the phosphorylation states of the peptide and PKB; if

18   the phosphorylation state of GSK3 is changed but that

19   of PKB is not then the substance being tested acts

20   directly on GSK3 without acting on PKB.

21   In a further aspect the present invention provides a

22   method of treatment of the human or non-human

23   (preferably mammalian) animal body, said method

24   comprising administering PKB, its analogues,

25   inhibitors, stimulators or functional equivalents

26   thereof to said body. Said method affects the

27   regulation of glycogen metabolism in the treated body.

28

29   The method of treatment of the present invention may be

30   of particular use in the treatment of type II diabetes

31   (where desirably an activator of PKB is used, so that

32   the down-regulation of GSK3 activity due to the action

33   of PKB is enhanced).

34

35   The method of treatment of the present invention may

36   alternatively be of particular use in the treatment of

cancer such as ovarian cancer (where desirably an
inhibitor of PKB is used, so that the down-regulation
of GSK3 activity due to the action of PKB is
depressed). Other cancers associated with
irregularities in the activity of PKB and/or GSK3 may
also be treated by the method, such as pancreatic
cancer, and breast cancer.

Stimulation of PKB with insulin increases activity
12-fold within 5 min and induces its phosphorylation at
Thr-308 and Ser-473. PKB transiently transfected into
cells can be activated 20-fold in response to insulin
and 46-fold in response to IGF-1 and also became
phosphorylated at Thr-308 and Ser-473. The activation
of PKB and its phosphorylation at both Thr-308 and
Ser-473 can be prevented by the phosphatidylinositol
(Pl) 3-kinase inhibitor wortmannin. The
phosphorylation of threonine 308 and serine 473 act
synergistically to activate PKB.

MAPKAP kinase-2-phosphorylated PKB at Ser-473 in vitro
increases activity seven-fold, an effect that can be
mimicked (fivefold activation) by mutating Ser-473 to
Asp. Mutation of Thr-308 to Asp also increases PKB
activity five-fold and subsequent phosphorylation of
Ser-473 by MAPKAP kinase-2 stimulates activity a
further fivefold, an effect mimicked (18-fold
activation) by mutating both Thr-308 and Ser-473 to
Asp. The activity of the Asp-308/Asp-473 double mutant
was similar to that of the fully phosphorylated enzyme
and could not be activated further by insulin. Mutation
of Thr-308 to Ala did not prevent the phosphorylation
of transfected PKB at Ser-473 after stimulation of 293
cells with insulin or IGF-1, but abolished the
activation of PKB. Similarly, mutation of Ser-473 to
Ala did not prevent the phosphorylation of transfected

1 PKB at Thr-308 but greatly reduced the activation of
2 transfected PKB. This demonstrates that the activation
3 of PKB by insulin or IGF-1 results from the
4 phosphorylation of Thr-308 and Ser-473 and that
5 phosphorylation of both residues is preferred to
6 generate a high level of PKB activity in vitro or in
7 vivo. Also, phosphorylation of Thr-308 in vivo is not
8 dependent on the phosphorylation of Ser-473 or vice
9 versa, that the phosphorylation of Thr-308 and Ser-473
10 are both dependent on PI 3-kinase activity and suggest
11 that neither Thr-308 nor Ser-473 phosphorylation is
12 catalysed by PKB itself.
13
14 Thus, it is preferred that the present invention
15 incorporates the use of any agent which affects
16 phosphorylation of PKB at amino acids 308 and/or 473,
17 for example insulin, inhibitors of PI 3-kinase such as
18 wortmannin or the like. The use of PKB, itself altered
19 at amino acids 308 and/or 473 (eg by phosphorylation
20 and/or mutation) is also suitable.
21
22 In a variation of the method of the present invention,
23 stimulation or inhibition of PKB may be assessed by
24 monitoring the phosphorylation states of amino acids
25 308 and/or 473 on PKB itself.
26
27 Different isoforms of PKB may be used or targeted in
28 the present invention; for example PKBα, β or γ.
29
30 The present invention will now be described in more
31 detail in the accompanying examples which are provided
32 by way of non-limiting illustration, and with reference
33 to the accompanying drawings.
34
35 **Example 1:PKB influences GSK3 activity.**
36 *Fig 1:* a, L6 myotubes were incubated for 15 min with 2

1   mM 8-bromocyclic-AMP (8Br-cAMP) and then with 0.1 μM
2   insulin (5 min). Both GSK3 isoforms were co-
3   immunoprecipitated from the lysates and assayed before
4   (black bars) and after (white bars) reactivation with
5   PP2A (Cross et al., 1994). The results are presented
6   relative to the activity in unstimulated cells, which
7   was 0.08±0.006 U mg^{-1} (n=10).
8   b, c, The inhibition of GSK3 by insulin (0.1 μM) is
9   unaffected by rapamycin (0.1 μM) and PD 98059 (50 μM),
10 but prevented by LY 294002 (100 μM).
11
12 b, L6 myotubes were stimulated with insulin for the
13 times indicated with (filled triangle) or without
14 (filled circles) a 15 min preincubation with LY 294002,
15 and GSK3 measured as in a. The open circles show
16 experiments from insulin-stimulated cells where GSK3
17 was assayed after reactivation with PP2A (Cross et al.,
18 1994).
19
20 c, cells were incubated with rapamycin (triangles) or
21 rapamycin plus PD 98059 (circles) before stimulation
22 with insulin, and GSK3 activity measured as in a,
23 before (filled symbols) and after (open symbols)
24 pretreatment with PP2A.
25
26 d, e, L6 myotubes were incubated with 8Br-cAMP (15 min)
27 PD 98059 (60 min) or LY 294002 (15 min) and then with
28 insulin (5 min) as in a-c. Each enzyme was assayed
29 after immunoprecipitation from lysates, and the results
30 are presented relative to the activities obtained. In
31 the presence of insulin and absence of 8Br-cAMP, which
32 were 0.04±0.005 U mg^{-1} (p42 MAP kinase, n=6) and 0.071 ±
33 0.004 U mg^{-1} (MAPKAP Kinase^{-1}, n=6).
34
35 All the results (± s.e.m.) are for at least three
36 experiments.

1   Monolayers of L6 cells were cultured, stimulated and
2   lysed as described previously (Cross et al., 1994).
3   p42 MAP kinase, MAPKAP kinase 1 or (GSK3-α plus GSK3-β)
4   were then immunoprecipitated from the lysates and
5   assayed with specific protein or peptide substrates as
6   described previously (Cross et al., 1994). One unit of
7   protein kinase activity was that amount which catalysed
8   the phosphorylation of 1 nmol of substrate in 1 min.
9   Where indicated, GSK3 in immunoprecipitates was
10 reactivated with PP2A (Cross et al., 1994).
11
12
13 **Figure 2 Identification of PKB as the insulin-**
14 **stimulated, wortmannin-sensitive and PD**
15 **98059/rapamycin-insensitive Crosstide kinase in L6**
16 **myotubes.**
17 a. Cells were incubated with 50 μM PD 98059 (for 1
18 hour) and 0.1 μM rapamycin (10 min), then stimulated
19 with 0.1 μM insulin (5 min) and lysed (Cross et al.,
20 1994). The lysates (0.3 mg protein) were
21 chromatographed on Mono Q (5 x 0.16cm) and fractions
22 (0.05ml) were assayed for Crosstide kinase (filled
23 circles). In separate experiments insulin was omitted
24 (open circles) or wortmannin (0.1 μM) added 10 min
25 before the insulin (filled triangles). The broken line
26 shows the NaCl gradient. Similar results were obtained
27 in six experiments.
28
29 b. Pooled fractions (10 μl) 31-34 (lane 1), 35-38 (lane
30 2), 39-42 (lane 3), 43-45 (lane 4), 46-49 (lane 5) and
31 50-53 (lane 6) from a were electrophoresed on a 10%
32 SDS/polyacrylamide gel and immunoblotted with the C-
33 terminal anti-PKBα antibody. Marker proteins are
34 indicated. No immunoreactive species were present in
35 fractions 1-30 or 54-80.
36

c. L6 myotubes were stimulated with 0.1 μM insulin and
PKB immunoprecipitated from the lysates (50 μg protein)
essentially as described previously (Lazar et al.,

4   1995), using the anti-PH domain antibody and assayed
5   for Crosstide kinase (open circles). In control
6   experiments, myotubes were incubated with 0.1 μM
7   rapamycin plus 50 μM PD 98059 (open triangles) or 2 mM
8   8Br-cAMP (open squares), or 0.1 μM wortmannin (filled
9   circles) or 100 μM LY 294002 (filled triangles) before
10   stimulation with insulin.
11
12   *d.* As *c*, except that MAPKAP kinase-1 was
13   immunoprecipitated from the lysates and assayed with S6
14   peptide (filled circles). In control experiments,
15   cells were incubated with 0.1 μM rapamycin plus 50 μM
16   PD 98059 (filled triangles) or with 2 μM 8BR-cAMP (open
17   circles) before stimulation with insulin. In *c* and *d*,
18   the error bars denote triplicate determinations, and
19   similar results were obtained in three separate
20   experiments.
21
22   Mono Q chromatography was performed as described
23   (Burgering et al., 1995), except that the buffer also
24   contained 1 mM EGTA, 0.1 mM sodium orthovanadate and
25   0.5% (w/v) Triton X-100. Two PKBα antibodies were
26   raised in rabbits against the C-terminal peptide
27   FPQFSYSASSTA and bacterially expressed PH domain of
28   PKBα. The C-terminal antibody was affinity purified
29   (Jones et al., 1991). The activity of PKB towards
30   Crosstide is threefold higher than its activity towards
31   histone H2B and 11-fold higher than its activity
32   towards myelin basic protein, the substrates used
33   previously to assay PKB. Other experimental details
34   and units of protein kinase activity are given in
35   Fig 1.
36

1   **Figure 3 GSK3 is inactivated by PKB from insulin-**
2   **stimulated L6 myotubes.**
3   *a.* Cells were stimulated for 5 min with 0.1 μM insulin,
4   and PKB immunoprecipitated from 100 μg of cell lysate
5   and used to inactivate GSK3 isoforms essentially as
6   described previously (Sutherland et al., 1993 and
7   Sutherland et al., 1994). The black bars show GSK3
8   activity measured after incubation with MgATP and PKB
9   as a percentage of the activity obtained in control
10 incubations where PKB was omitted. In the absence of
11 PKB, GSK3 activity was stable throughout the
12 experiment. The white bars show the activity obtained
13 after reactivation of GSK3 with PP2A (Embi et al.,
14 1980). No inactivation of GSK3 occurred if insulin was
15 omitted, or if wortmannin (0.1 μM) was added 10 min
16 before the insulin, or if the anti-PKB antibody was
17 incubated with peptide immunogen (0.5 mM) before
18 immunoprecipitation. The results ($\pm$ s.e.m.) are for
19 three experiments (each carried out in triplicate).
20
21 *b.* Inactivation of GSK3-β by HA-PKBα. Complementary
22 DNA encoding HA-PKBα was transfected into COS-1 cells,
23 and after stimulation for 15 min with 0.1 mM sodium
24 pervanadate the tagged protein kinase was
25 immunoprecipitated from 0.3 mg of lysate and incubated
26 for 20 min with GSK3-β and MgATP. In control
27 experiments, pervanadate was omitted, or wildtype (WT)
28 PKBα replaced by vector (mock translation) or by a
29 kinase-inactive mutant of PKBα in which Lys 179 was
30 mutated to Ala (K179A). Similar results were obtained
31 in three separate experiments. The levels of WT and
32 K179A-PKBα in each immunoprecipitate were similar in
33 each transfection.
34
35 In *a* GSK3-α and GSK3-β were partially purified,
36 assayed, inactivated by PKB, and reactivated by PP2A

1   from rabbit skeletal muscle as described previously
2   (Sutherland et al., 1993 and Sutherland et al.,
3   1994). There was no reactivation in control
4   experiments in which okadaic acid (2 μM) was added
5   before PP2A.

6

7   **Figure 4: Identification of the residues in GSK3**
8   **phosphorylated by PKB *in vitro* and in response to**
9   **insulin in L6 myotubes.**

10   *a*. GSK3-β was maximally inactivated by incubation with
11   PKB and Mg-[γ-^{32}P]ATP and after SDS-PAGE, the ^{32}P-
12   labelled GSK3-β (M_r 47K) was digested with trypsin[11] and
13   chromatographed on a C_{18} column (Sutherland et al.,
14   1993). Fractions (0.8 ml) were analysed for ^{32}P-
15   radioactivity (open circles), and the diagonal line
16   shows the acetonitrile gradient.

17

18   *b*. The major phosphopeptide from a (400 c.p.m.) was
19   subjected to solid-phase sequencing (Sutherland et al.,
20   1993), and ^{32}P-radioactivity released after each cycle
21   of Edman degradation is shown.

22

23   *c*. GSK3-α and GSK3-β were co-immunoprecipitated from
24   the lysates of ^{32}P-labelled cells, denatured in SDS,
25   subjected to SDS-PAGE, transferred to nitrocellulose
26   and autoradiographed (Saito et al., 1994). Lanes 1-3,
27   GSK3 isoforms immunoprecipitated from unstimulated
28   cells; lanes 4-6, GSK3 isoforms immunoprecipitated from
29   insulin-stimulated cells.

30

31   *d*. GSK3 isoforms from *c*. were digested with trypsin,
32   and the resulting phosphopeptides separated by
33   isoelectric focusing (Saito et al., 1994) and
34   identified by auto-radiography. Lanes 1 and 4 show the
35   major phosphopeptide resulting from *in vitro*
36   phosphorylation of GSK3-β by PKB and MAPKAP kinase-1,

1   respectively; lanes 2 and 5, the phosphopeptides
2   obtained from GSK3-β and GSK3-α, immunoprecipitated
3   from unstimulated cells; lanes 3 and 6, the
4   phosphopeptides obtained from GSK3-β and GSK3-α
5   immunoprecipitated from cells stimulated for 5 min with
6   0.1 μM insulin; the arrow denotes the peptides whose
7   phosphorylation is increased by insulin. The pI values
8   of two markers, Patent Blue (2.4) and azurin (5.7) are
9   indicated.
10
11   In a. PKBα was immunoprecipitated with the C-terminal
12   antibody from the lysates (0.5 mg protein) of insulin-
13   stimulated L6 myotubes and used to phosphorylate GSK-
14   β[12]. In c. three 10-cm diameter dishes of L6 myotubes
15   were incubated for 4 hours in HEPES-buffered saline
16   (Cross et al., 1994) containing 50 μM PD 98059, 100 nM
17   rapamycin and 1.5 mCI ml^{-1} ^{32}P-orthophosphate, stimulated
18   for 5 min with insulin (0.1 μM) or buffer, and GSK3
19   isoforms co-immunoprecipitated from the lysates as in
20   Fig 1.
21
22   **Discussion.**
23   Inhibition of GSK3 induced by insulin in L6 myotubes
24   (Fig 1a-c) was unaffected by agents which prevented the
25   activation of MAPKAP kinase-1 (8-bromo-cyclic AMP, or
26   PD 98059 (Alessi et al., 1995), (Fig 1d,e) and/or p70^{S6k}
27   (rapamycin (Kuo et al., 1992)) (Cross et al., 1994),
28   suggesting that neither MAPKAP kinase-1 nor p70^{S6k} are
29   essential for this process. However, the
30   phosphorylation and inhibition of GSK3-β after phorbol
31   ester treatment (Stambolic et al., 1994) is enhanced by
32   coexpression with MAPKAP kinase 1 in HeLa S3 cells,
33   whereas in NIH 3T3 cells the EGF-induced inhibition of
34   GSK3-α and GSK3-β (Saito et al., 1994) is largely
35   suppressed by expression of a dominant-negative mutant
36   of MAP kinase kinase-1 (Elgar et al., 1995). MAPKAP

1   kinase-1 may therefore mediate the inhibition of GSK3

2   by agonists which are much more potent activators of

3   the classical MAP kinase pathway than is insulin.

4

5   To identify the insulin-stimulated protein kinase that

6   inhibits GSK3 in the presence of rapamycin and PD

7   98059, L6 myotubes were incubated with both compounds

8   and stimulated with insulin. The lysates were then

9   chromatographed on Mono Q and the fractions assayed

10   with "Crosstide" (GRPRTSSFAEG), a peptide corresponding

11   to the sequence in GSK3 surrounding the serine

12   (underlined) phosphorylated by MAPKAP kinase-1 and $p70^{S6k}$

13   (Ser 21 in GSK3-α (Sutherland et al., 1994) and Ser 9

14   in GSK3-β (Sutherland et al 1993)). Three peaks of

15   Crosstide kinase activity were detected, which were

16   absent if insulin stimulation was omitted or if the

17   cells were first preincubated with the PI 3-kinase

18   inhibitor wortmannin (Fig 2a). Wortmannin (Cross et

19   al., 1994 and Welsh et al 1994), and the structurally

20   unrelated PI 3-kinase inhibitor LY 294002 (ref 19);

21   (Fig 1b), both prevent the inhibition of GSK3 by

22   insulin.

23

24   The protein kinases PKB-α, PKB-β and PKBγ are Ser/Thr-

25   specific and cellular homologues of the viral oncogene

26   v-akt (Coffer et al., 1991, Jones et al 1991, Ahmed et

27   al 1995 and Cheng et al., 1992). These enzymes have

28   recently been shown to be activated in NIH 3T3, Rat-1

29   or Swiss 3T3 cells in response to growth factors or

30   insulin, activation being suppressed by blocking the

31   activation of PI 3-kinase in different ways (Franke et

32   al., 1995 and Burgering et al., 1995). All three peaks

33   of Crosstide kinase (Fig 2a), but no other fraction

34   from Mono Q, showed the characteristic multiple bands

35   of PKB (relative molecular mass, M_r 58K, 59K or 60K)

36   that have been observed in other cells, when

1   immunoblotting was performed with an antibody raised

2   against the carboxyl-terminal peptide of PKB-α (Fig

3   2b). The more slowly migrating forms represent more

4   highly phosphorylated protein, and are converted to the

5   fastest migrating species by treatment with

6   phosphatases. Phosphatase treatment also results in

7   the inactivation of PKB (Burgering et al., 1995) and

8   the complete loss of Crosstide kinase activity (data

9   not shown). Of the Crosstide kinase activity in peaks

10 2 and 3 from Mono Q, 70-80% was immunoprecipitated by a

11 separate antibody raised against the amino-terminal

12 pleckstrin homology (PH) domain of PKB-α. The C-

13 terminal antibody also immunoprecipitated PKB activity

14 specifically from peaks 2 and 3, but was less effective

15 than the anti-PH-domain antibody. Peak-1 was hardly

16 immunoprecipitated by either antibody and may represent

17 PKB-β. An immunoprecipitating anti-MAPKAP kinase-1

18 antibody (Cross et al., 1994) failed to deplete any of

19 the Crosstide kinase activity associated with peaks 1,

20 2 or 3.

21

22 Insulin stimulation of L6 myotubes increased PKB

23 activity by more than tenfold (Fig 2c), and activation

24 was blocked by wortmannin or LY 294002, but was

25 essentially unaffected by 8-bromo-cyclic AMP or

26 rapamycin plus PD 98059 (Fig 2c). The half-time ($t_{0.5}$)

27 or activation of PKB (1 min) was slightly faster than

28 that for inhibition of GSK3 (2 min) (Cross et al.,

29 1994). In contrast, the activation of MAPKAP kinase-1

30 (Fig 2d) and p70^{S6k} (not shown) was slower ($t_{0.5}$ > 5 min).

31 Activation of MAPKAP kinase-1 was prevented by 8-bromo-

32 cyclic AMP or PD 98059 (Fig 2d), and activation of p70^{S6k}

33 by rapamycin (Cross et al., 1994). Akt/RAC

34 phosphorylated the Ser in the Crosstide equivalent to

35 Ser 21 in GSK3-α and Ser 9 in GSK3-β (data not shown).

36

PKB from insulin-stimulated L6 myotubes (but not from
unstimulated or wortmannin-treated cells) inactivated
GSK3-α and GSK3-β in vitro, and inhibition was reversed
by the Ser/Thr-specific protein phosphatase PP2A (Embi
et al., 1980) (Fig 3a). To further establish that
inactivation was catalysed by PKB, and not by a co-
immunoprecipitating protein kinase, haemagglutonin-
tagged PKB-α (HA-PKB) was transfected into COS-1 cells
and activated by stimulation with pervanadate, which is
the strongest inducer of PKB activation in this system.
The HA-PKB inactivated GSK3-β, but not if treatment
with pervanadate was omitted or if wild-type HA-PKB was
replaced with a "kinase inactive" mutant (Fig 3b).

The inactivation of GSK3-β by PKB in vitro was
accompanied by the phosphorylation of one major tryptic
peptide (Fig 4a) which coeluted during C_{18}
chromatography (Sutherland et al., 1993) and
isoelectric focusing with that obtained after
phosphorylation by MAPKAP kinase-1 (Fig 4d).
Stimulation of L6 myotubes with insulin (in the
presence of rapamycin and PD 98059) increased the ^{32}P-
labelling of GSK3-α and GSK3-β by 60-100% (Fig 4c) and
increased the ^{32}P-labelling of the same tryptic peptides
labelled in vitro (Fig 4d). Sequence analyses
established that the third residue of these,
corresponding to Ser 9 (GSK3-β) or Ser 21 (GSK3-α), was
the site of phosphorylation in each phosphopeptide,
both in vitro (Fig 4b) and in vivo (not shown). The
^{32}P-labelling of other (more acidic) tryptic
phosphopeptides was not increased by insulin (Fig 4d).
These peptides have been noted previously in GSK3 from
A431 cells and shown to contain phosphoserine and
phosphotyrosine (Saito et al., 1994).

PKC-δ, ε and ζ are reported to be activated by

1   mitogens, and PKC-ζ activity is stimulated in vitro by
2   several inositol phospholipids, including $PI(3,4,5)P_3$
3   the product of the PI 3-kinase reaction (Andjelkovic et
4   al., 1995). However, purified PKC-ε (Palmer et al.,
5   1995), PKC-δ and PKC-ζ (data not shown) all failed to
6   inhibit GSK3-α or GSK3-β in vitro. Moreover, although
7   PKC-α, β1 and γ inhibit GSK3-β in vitro (Palmer et al.,
8   1995), GSK3-α is unaffected, while their downregulation
9   in L6 myotubes by prolonged incubation with phorbol
10 esters abolishes the activation of MAPKAP kinase-1 in
11 response to subsequent challenge with phorbol esters,
12 but has no effect on the inhibition of GSK3 by insulin
13 (not shown).
14
15 Taken together, our results identify GSK3 as a
16 substrate for PKB. The stimulation of glycogen
17 synthesis by insulin in skeletal muscle involves the
18 dephosphorylation of Ser residues in glycogen synthase
19 that are phosphorylated by GSK3 in vitro (Parker et
20 al., 1983). Hence the 40-50% inhibition of GSK3 by
21 insulin, coupled with a similar activation of the
22 relevant glycogen synthase phosphatase (Goode et al.,
23 1992), can account for the stimulation of glycogen
24 synthase by insulin in skeletal muscle (Parker et al.,
25 1983) or L6 myotubes (Goode et al., 1992). The
26 activation of glycogen synthase and the resulting
27 stimulation of glycogen synthesis by insulin in L6
28 myotubes is blocked by wortmannin, but not by PD 98059
29 (Dent et al., 1990), just like the activation of
30 Akt/RAC and inhibition of GSK3. However, GSK3 is
31 unlikely to be the only substrate of PKB in vivo, and
32 identifying other physiologically relevant substrates
33 will be important because PKBβ is amplified and over-
34 expressed in many ovarian neoplasms (Cheng et al.,
35 1992).
36

19

1   **Example 2: Activation of PKB by insulin in L6 myotubes**
2   **is accompanied by phosphorylation of residues Thr-308**
3   **and Ser-473.**   Insulin induces the activation and
4   phosphorylation of PKBα in L6 myotubes. Three 10 cm
5   dishes of L6 myotubes were ^{32}P-labelled and treated for
6   10 min with or without 100 nM wortmannin and then for 5
7   min with or without 100 nM insulin. PKBα was
8   immunoprecipitated from the lysates and an aliquot
9   (15%) assayed for PKBα activity (Fig 5A). The
10 activities are plotted ± SEM for 3 experiments relative
11 to PKBα derived from unstimulated cells which was 10
12 mU/mg. The remaining 85% of the immunoprecipitated PKBα
13 was alkylated with 4-vinylpyridine, electrophoresed on
14 a 10% polyacrylamide gel (prepared without SDS to
15 enhance the phosphorylation-induced decrease in
16 mobility) and autoradiographed. The positions of the
17 molecular mass markers glycogen phosphorylase (97 kDa),
18 bovine serum albumin (66 kDa) and ovalbumin (43 kDa)
19 are marked.
20
21 Under these conditions, insulin stimulation resulted in
22 a 12-fold activation of PKBα (Fig 5A) and was
23 accompanied by a 1.9 ± 0.3-fold increase in
24 ^{32}P-labelling (4 experiments) and retardation of its
25 mobility on SDS-polyacrylamide gels (Fig 5B). The
26 activation of PKBα, the increase in its ^{32}P-labelling
27 and reduction in electrophoretic migration were all
28 abolished by prior incubation of the cells with 100 nM
29 wortmannin. Phosphoamino acid analysis of the whole
30 protein revealed that ^{32}P-labelled PKBα was
31 phosphorylated at both serine and threonine residues
32 and that stimulation with insulin increased both the
33 ^{32}P-labelling of both phosphoamino acids (data not
34 shown).
35
36 **Fig. 6. Insulin stimulation of L6 myotubes induces the**

1   **phosphorylation of two peptides in PKBα.**   Bands
2   corresponding to ^{32}P-labelled PKBα from Fig 5B were
3   excised from the gel, treated with 4-vinylpyridine to
4   alkylate cysteine residues, digested with trypsin and
5   chromatographed on a Vydac 218TP54 C18 column
6   (Separations Group, Hesperia, CA) equilibrated with
7   0.1% (by vol) trifluoroacetic acid (TFA), and the
8   columns developed with a linear acetonitrile gradient
9   (diagonal line). The flow rate was 0.8 ml / min and
10   fractions of 0.4 ml were collected (A) tryptic peptide
11   map of ^{32}P-labelled PKBα from unstimulated L6 myotubes;
12   (B) tryptic peptide map of ^{32}P-labelled PKBα from
13   insulin-stimulated L6 myotubes; (C) tryptic peptide map
14   of ^{32}P-labelled PKBα from L6 myotubes treated with
15   wortmannin prior to insulin. The two major ^{32}P-labelled
16   peptides eluting at 23.7% and 28% acetonitrile are
17   named Peptide A and Peptide B, respectively. Similar
18   results were obtained in four (A and B) and two (C)
19   experiments.
20
21   No major ^{32}P-labelled peptides were recovered from
22   ^{32}P-labelled PKBα derived from unstimulated L6 myotubes
23   (Fig 6A) indicating that, in the absence of insulin,
24   there was a low level phosphorylation at a number of
25   sites. However, following stimulation with insulin, two
26   major ^{32}P-labelled peptides were observed, termed A and
27   B (Fig 6B), whose ^{32}P-labelling was prevented if the
28   myotubes were first preincubated with wortmannin (Fig
29   6C).
30
31   **Fig 7. Identification of the phosphorylation sites in**
32   **peptides A and B.** (A) Peptides A and B from Fig5B
33   (1000cpm) were incubated for 90min at 110°C in 6M HCl,
34   electrophoresed on thin layer cellulose at pH 3.5 to
35   resolve orthophosphate (Pi), phosphoserine (pS),
36   phosphthreonine (pT) and phosphotyrosine (pY) and

1   autoradiographed. (B) Peptide A (Fig 5B) obtained from

2   50 10 cm dishes of ^{32}P-labelled L6 myotubes was further

3   purified by chromatography on a microbore C18 column

4   equilibrated in 10 mM ammonium acetate pH 6.5 instead

5   of 0.1% TFA. A single peak of ^{32}P-radioactivity was

6   observed at 21% acetonitrile which coincided with a

7   peak of 214 nm absorbance. 80% of the sample (1 pmol)

8   was analysed on an Applied Biosystems 476A sequencer to

9   determine the amino acid sequence, and the

10   phenylthiohydantoin (Pth) amino acids identified after

11   each cycle of Edman degradation are shown using the

12   single letter code for amino acids. The residues in

13   parentheses were not present in sufficient amounts to

14   be identified unambiguously. To identify the site(s)

15   of phosphorylation, the remaining 20% of the sample

16   (600 cpm) was then coupled covalently to a Sequelon

17   arylamine membrane and analysed on an Applied

18   Biosystems 470A sequencer using the modified programme

19   described by Stokoe et al. (1992). ^{32}P radioactivity was

20   measured after each cycle of Edman degradation. (C)

21   Peptide B from Fig 2B (800 cpm) was subjected to solid

22   phase sequencing as in (B).

23

24   Peptide A was phosphorylated predominantly on serine

25   while peptide B was labelled on threonine (Fig 7A).

26   Amino acid sequencing established that peptide A

27   commenced at residue 465. Only a single burst of

28   ^{32}P-radioactivity was observed after the eighth cycle of

29   Edman degradation (Fig 7B), demonstrating that insulin

30   stimulation of L6 myotubes had triggered the

31   phosphorylation of PKBα at Ser-473, which is located 9

32   residues from the C-terminus of the protein.

33   Phosphopeptide B was only recovered in significant

34   amounts if ^{32}P-labelled PKBα was treated with

35   4-vinylpyridine prior to digestion with trypsin,

36   indicating that this peptide contained a cysteine

1   residue(s), and a single burst of 32p radioactivity was

2   observed after the first cycle of Edman degradation

3   (Fig 7C). This suggested that the site of

4   phosphorylation was residue 308, since it is the only

5   threonine in PKBa that follows a lysine or arginine

6   residue and which is located in a tryptic peptide

7   containing a cysteine residue (at position 310). The

8   acetonitrile concentration at which phosphopeptide B is

9   eluted from the C18 column (28%) and its isoelectric

10 point (4.0) are also consistent with its assignment as

11 the peptide comprising residues 308-325 of PKBα. The

12 poor recoveries of Peptide B during further

13 purification at pH 6.5 prevented the determination of

14 its amino acid sequence, but further experiments

15 described below using transiently transfected 293 cells

16 established that this peptide does correspond to

17 residues 308-325 of PKBα.

18

19 **Fig 8: Mapping the phosphorylation sites of PKBα in**

20 **transiently transfected 293 cells.** 293 cells were

21 transiently transfected with DNA constructs expressing

22 wild type PKBα, or a haemagglutonin epitope-tagged PKBα

23 encoding the human protein, such as HA-KD PKBα, HA-473A

24 PKBα or HA-308A PKBα. After treatment for 10 min with

25 or without 100 nM wortmannin, the cells were stimulated

26 for 10 min with or without 100 nM insulin or 50 ng/ml

27 IGF- 1 in the continued presence of wortmannin. PKBα

28 was immunoprecipitated from the lysates and assayed,

29 and activities corrected for the relative levels of

30 expression of each HA-PKBα. The results are expressed

31 relative to the specific activity of wild type HA-PKBα

32 from unstimulated 293 cells (2.5 ± 0.5 U/mg). (B) 20 μg

33 of protein from each lysate was electrophoresed on a 10

34 % SDS/polyacrylamide gel and immunoblotted using

35 monoclonal HA-antibody. The molecular markers are those

36 used in Fig 5B.

1   **Fig 9: IGF-1 stimulation of 293 cells induces the**

2   **phosphorylation of two peptides in transfected HA-PKBα.**

3   293 cells transiently transfected with wild type HAPKBα

4   DNA constructs were ^{32}P-labelled, treated for 10 min

5   without (A,B) or with (C) 100 nM wortmannin and then

6   for 10 min without (A) or with (B, C) 50 ng/ml IGF-1.

7   The 32p labelled HA-PKBα was immunoprecipitated from

8   the lysates, treated with 4-vinylpyridine,

9   electrophoresed on a 10% polyacrylamide gel, excised

10   from the gel and digested with trypsin. Subsequent

11   chromatography on a C_{18} column resolved four major

12   phosphopeptides termed C, D, E and F. Similar results

13   were obtained in 6 separate experiments for (A) and

14   (B), and in two experiments for (C).

15

16   Stimulation with insulin and IGF-1 resulted in 20-fold

17   and 46-fold activation of transfected PKBα,

18   respectively (Fig 8A), the half time for activation

19   being 1 min, as found with other cells. Activation of

20   PKBα by insulin or IGF-1 was prevented by prior

21   incubation with wortmannin (Fig 8A) and no activation

22   occurred if 293 cells were transfected with vector

23   alone and then stimulated with insulin or 1GF-1 (data

24   not shown).

25

26   Two prominent ^{32}P-labelled peptides were present in

27   unstimulated 293 cells (Fig 9A). One, termed Peptide C,

28   usually eluted as a doublet at 20-21% acetonitrile

29   while the other, termed Peptide F, eluted at 29.7%

30   acetonitrile. Stimulation with insulin or IGF-1 did

31   not affect the ^{32}P-labelling of Peptides C and F (Figs

32   9A & B), but induced the ^{32}P-labelling of two new

33   peptides, termed D (23.4% acetonitrile) and E

34   (28% acetonitrile), which eluted at the same

35   acetonitrile concentrations as peptides A and B from L6

36   myotubes (Fig 6B) and had the same isoelectric points

24

(7.2 and 4.0, respectively). Treatment of 293 cells expressing HA-PKBα with 100 nM wortmannin, prior to stimulation with IGF-1, prevented the phosphorylation of Peptides D and E, but had no effect on the 32p labelling of Peptides C and F (Fig 9C).

Peptides C, D, E and F were further purified by re-chromatography on the C18 column at pH 6.5 and sequenced. Peptides C gave rise to three separate (but closely eluting) ^{32}P-labelled peptides (data not shown). Amino acid sequencing revealed that all three commenced at residue 122 of PKBα and that Ser-124 was the site of phosphorylation (Fig 10A). Peptide D only contained phosphoserine and, as expected, corresponded to the PKBα tryptic peptide commencing at residue 465 that was phosphorylated at Ser-473 (Fig 10B). Peptide E, only contained phosphothreonine and amino acid sequencing demonstrated that it corresponded to residues 308-325, the phosphorylation site being Thr-308 (Fig 10C). Peptide F only contained phosphothreonine and corresponded to the peptide commencing at residue 437 of PKBα phosphorylated at Thr-450 (Fig 10D).

In the presence of phosphatidylserine, PKBα binds to PIP3 with submicromolar affinity (James et al., 1996, Frech et al.,1996). Phosphatidyl 4,5-bisphosphate and phosphatidyl 3,4 bisphosphate bind to PKBα with lower affinities and PI 3,5 bisphosphate and PI 3 phosphate did not bind at all under these conditions (James et al., 1996). The region of PKBα that interacts with PIP3 is almost certainly the PH domain, because the isolated PH domain binds PIP3 with similar affinity to PKBα itself (Frech et al., 1996) and because the PH domain of several other proteins, such as the PH-domains of, β-spectrin and phospholipase C1, are known to interact specifically with other phosphoinositides (Hyvonen et

1   al., 1995 and Lemmon et al., 1995).

2

3   The experiments described above were repeated using

4   insulin instead of IGF-1. The results were identical,

5   except that the ^{32}P-labelling of Peptides D and E was

6   about 50% of the levels observed with IGF-1 (data not

7   shown). This is consistent with the two-fold lower

8   level of activation of PKBα by insulin compared with

9   IGF-1 (Fig 7A).

10

11   **Example 3: MAPKAP kinase-2 phosphorylates Ser-473 of**

12   **PKBα causing partial activation.** Ser-473 of PKBa lies

13   in a consensus sequence Phe-x-x-Phe/Tyr-Ser/Thr-Phe/Tyr

14   found to be conserved in a number of protein kinases

15   that participate in signal transduction pathways

16   (Pearson et al. 1995). In order to identify the Ser-473

17   kinase(s) we therefore chromatographed rabbit skeletal

18   muscle extracts on CM-Sephadex, and assayed the

19   fractions for protein kinases capable of

20   phosphorylating a synthetic peptide corresponding to

21   residues 465 to 478 of PKBα. These studies identified

22   an enzyme eluting at 0.3 M NaCl which phosphorylated

23   the peptide 465-478 at the residue equivalent to

24   Ser-473 of PKBα. The Ser473 kinase co-eluted from

25   CM-Sephadex with MAP kinase-activated protein (MAPKAP)

26   kinase-2, (Stokoe et al, 1992) which is a component of

27   a stress and cytokine-activated MAP kinase cascade

28   (Rouse et al, 1994; Cuenda et al, 1995). The Ser-473

29   kinase continued to cofractionate with MAPKAPkinase-2

30   through phenyl-Sepharose, heparin-Sepharose, Mono S and

31   Mono Q and was immunoprecipitated quantitatively by an

32   anti-MAPKAP kinase-2 antibody (Gould et al, 1995)

33   demonstrating that MAPKAP kinase-2 was indeed the

34   Ser-473 kinase we had purified.

35

36   **Figure 11.** HA-PKBα was immunoprecipitated from the

1   lysates of unstimulated COS-1 cells expressing these

2   constructs. (A) 0.5 μg of immunoprecipitated HA-PKBα

3   was incubated with MAPKAP kinase-2 (50 U/ml), 10 mM

4   magnesium acetate and 100 mM [γ^{32}P]ATP in a total of 40

5   μl of Buffer B. At various times, aliquots were removed

6   and either assayed for PKBα activity (open circles) or

7   for incorporation of phosphate into PKBα (closed

8   circles). Before measuring PKBa activity, EDTA was

9   added to a final concentration of 20 mM to stop the

10   reaction, and the immunoprecipitates washed twice with

11   1.0 ml of buffer B containing 0.5 M NaCl, then twice

12   with 1.0 ml of Buffer B to remove MAPKAP kinase-2. The

13   results are presented as $\pm$ SEM for six determinations

14   (two separate experiments) and PKBα activities are

15   presented relative to control experiments in which

16   HA-PKBα was incubated with MgATP in the absence of

17   MAPKAP kinase-2 (which caused no activation).

18   Phosphorylation was assessed by counting the

19   ^{32}P-radioactivity associated with the band of PKBα after

20   SDS/polyacrylamide gel electrophoresis. The open

21   triangles show the activity of immunoprecipitated HA-KD

22   PKBα phosphorylated by MAPKAP kinase-2. (B) HA-PKBα

23   phosphorylated for 1 h with MAPKAP kinase-2 and ^{32}P-

24   γ-ATP as in (A) was digested with trypsin and

25   chromatographed on a C18 column as described in the

26   legend for Fig 2. (C) The major ^{32}P-labelled peptide

27   from (B) was analysed on the 470A sequencer as in Fig 3

28   to identify the site of phosphorylation.

29

30   Bacterially expressed MAPKAP kinase-2 phosphorylated

31   wild type HA-PKBα or the catalytically inactive mutant

32   HA-PKBα in which Lys- 179 had been mutated to Ala (data

33   not shown) to a level approaching 1 mol per mole

34   protein (Fig 11A). Phosphorylation of wild-type PKBα

35   was paralleled by a seven-fold increase in activity,

36   whereas phosphorylation of the catalytically inactive

1   mutant did not cause any activation (Fig 11A). No
2   phosphorylation or activation of wild type HA-PKBα
3   occurred if MAPKAP kinase-2 or MgATP was omitted from
4   the reaction (data not shown). Wild type HA-PKBα that
5   had been maximally activated with MAPKAP kinase-2, was
6   completely dephosphorylated and inactivated by
7   treatment with protein phosphatase 2A (data not shown).
8
9   HA-PKBα that had been maximally phosphorylated with
10   MAPKAP kinase-2 was digested with trypsin and C18
11   chromatography revealed a single major ^{32}P-labelled
12   phosphoserine-containing peptide (Fig 11B). This
13   peptide eluted at the same acetonitrile concentration
14   (Fig 11B) and had the same isoelectric point of 7.2
15   (data not shown) as the 32p labelled tryptic peptide
16   containing Ser-473 (compare Figs 11B and 6B). Solid
17   phase sequencing gave a burst of ^{32}P-radioactivity after
18   the eighth cycle of Edman degradation (Fig 11C),
19   establishing that Ser-473 was the site of
20   phosphorylation. The same ^{32}P-peptide was obtained
21   following tryptic digestion of catalytically inactive
22   HA-KD PKBα that had been phosphorylated with MAPKAP
23   kinase-2 (data not shown).
24
25   **Example 4: Phosphorylation of Thr-308 and Ser-473**
26   **causes synergistic activation of PKBα.** The experiments
27   described above demonstrated that phosphorylation of
28   Ser-473 activates PKBα in vitro but did not address the
29   role of phosphorylation of Thr-308, or how
30   phosphorylation of Thr-308 might influence the effect
31   of Ser-473 phosphorylation on activity, or vice versa.
32   We therefore prepared haemagglutonin (HA)-tagged PKBα
33   DNA constructs in which either Ser-473 or Thr-308 would
34   be changed either to Ala (to block the effect of
35   phosphorylation) or to Asp (to try and mimic the effect
36   of phosphorylation).

1   **Fig 12. Activation of HA-PKBα mutants in vitro by**
2   **MAPKAP kinase-2.** (A) Wild type and mutant HA-PKBα
3   proteins were immunoprecipitated from the lysates of
4   unstimulated COS-1 cells expressing these constructs
5   and incubated for 60 min with MgATP in the absence (-,
6   filled bars) or presence (+, hatched bars) of MAPKAP
7   kinase-2 and MgATP (50 U/ml). The PKBα protein was
8   expressed as similar levels in each construct and
9   specific activities are presented relative to wild type
10   HA-PKBα incubated in the absence of MAPKAP kinase-2
11   (0.03 U/mg). The results are shown as the average ± SEM
12   for 3 experiments. (B) 20 μg of protein from each
13   lysate was electrophoresed on a 10 % SDS/polyacrylamide
14   gel and immunoblotted using monoclonal HA-antibody.

15

16   All the mutants were expressed at a similar level in
17   serum-starved COS-1 cells (data not shown) and the
18   effects of maximally phosphorylating each of them at
19   Ser-473 is shown in Fig 12A. Before phosphorylation
20   with MAPKAP kinase-2 the activity of HA-473A PKBα was
21   similar to that of unstimulated wild type HA-PKBα and,
22   as expected, incubation with MAPKAP kinase-2 and MgATP
23   did not result in any further activation of HA-473A
24   PKBα. In contrast, the activity of HA-473D PKBα was
25   five-fold to six-fold higher than that of unstimulated
26   wild type HAPKBα protein, and similar to that of
27   wild-type HA-PKBα phosphorylated at Ser-473. As
28   expected, HA-473D PKBα was also not activated further
29   by incubation with MAPKAP kinase-2 and MgATP. The
30   activity of HA-308A PKBα was about 40% that of the
31   unstimulated wild type enzyme, and after
32   phosphorylation with MAPKAP kinase-2 is activity
33   increased to a level similar to that of wild type
34   HA-PKBα phosphorylated at Ser-473. Interestingly,
35   HA-308D PKBα which (like HA-473D PK) was five-fold more
36   active than dephosphorylated wild type HA-PKBα, was

1   activated dramatically by phosphorylation of Ser-473.
2   After incubation with MAPKAP kinase-2 and MgATP, the
3   activity of HA-308D PKBα was nearly five-fold higher
4   than that of wild type HA-PKBα phosphorylated at
5   Ser-473 (Fig 12B). These results suggested that the
6   phosphorylation of either Thr-308 or Ser-473 leads to
7   partial activation of PKBα in vitro, and that
8   phosphorylation of both residues results in a
9   synergistic activation of the enzyme. This idea was
10 supported by further experiments in which both Thr-308
11 and Ser-473 were changed to Asp. When this double
12 mutant was expressed in COS-1 cells it was found to
13 possess an 18-fold higher specific activity than the
14 dephosphorylated wild type protein. As expected, the
15 activity of this mutant was not increased further by
16 incubation with MAPKAP kinase-2 and MgATP (Fig 12B).
17
18 **Example 5:   Phosphorylation of both Thr-308 and Ser-473**
19 **is required for a high level of activation of PKBα in**
20 **vivo.**
21
22 **Fig 9. Effect of mutation of PKBα on its activation by**
23 **insulin in 293 cells.** (A) 293 cells were transiently
24 transfected with DNA constructs expressing wild type
25 PKBa, HA-D473- PKBα, and HA-308D/473D-PKBα. After
26 treatment for 10 min with or without 100 nM wortmannin,
27 cells were stimulated for 10 min with or without 100 nM
28 insulin in the continued presence of wortmannin. PKBα
29 was immunoprecipitated from the lysates and assayed,
30 and activities corrected for the relative levels of
31 HA-PKBα expression as described in the methods. The
32 results are expressed relative to the specific activity
33 of wild type HA-PKBα obtained from unstimulated 293
34 cells. (B) 20 μg of protein from each lysate was
35 electrophoresed on a 10 % SDS/polyacrylamide gel and
36 immunoblotted using monoclonal HA-antibody.

1   The basal level of activity of HA-473A PKBα derived
2   from unstimulated cells was similar to that of wild
3   type PKBα (Fig 8A). Stimulation of 293 cells expressing
4   HA-473A PKBα with insulin or IGF-1 increased the
5   activity of this mutant three-fold and five-fold
6   respectively; i.e. to 15% of the activity of wild type
7   HA-PKBα which had been transiently expressed and
8   stimulated under identical conditions. The basal
9   activity of HA-308A PKBα in unstimulated cells was also
10 similar to that of wild type HA-PKBα derived from
11 unstimulated cells, but virtually no activation of this
12 mutant occurred following stimulation of the cells with
13 insulin or IGF-1. These data are consistent with in
14 vitro experiments and indicate that maximal activation
15 of PKBα requires phosphorylation of both Ser-473 and
16 Thr-308 and that phosphorylation of both residues
17 results in a synergistic activation of the enzyme.
18 Consistent with these results, HA-473D PKBα displayed
19 five-fold higher activity and the HA-308D/HA473D double
20 mutant 40-fold higher activity than wild type HA-PKBα
21 when expressed in unstimulated cells. Following
22 stimulation with insulin, HA-473D PKBα was activated to
23 a level similar to that observed with the wildtype
24 enzyme, while the HA-308D/HA-473D double mutant could
25 not be activated further (Fig 13). As expected,
26 activation of HA-473D PKBα by insulin was prevented by
27 wortmannin, and the activity of the HA-308D/ HA-473D
28 double mutant was resistant to wortmannin (Fig 13).
29
30 Example 6: Phosphorylation of Thr-308 is not dependent
31 on phosphorylation of Ser-473 or vice versa (in 293
32 cells). (Fig 10) A 10 cm dish of 293 cells were
33 transfected with either HA-308A PKBα or HA-473A PKBα,
34 ^{32}P-labelled, then stimulated for 10 min with either
35 IGF-1 (50 ng/ml) or buffer. The ^{32}P-labelled PKBα
36 mutants were immunoprecipitated from the lysates,

1   treated with 4-vinylpyridine, electrophoresed on a 10%
2   polyacrylamide gel, excised from the gel and digested
3   with trypsin, then chromatographed on a C18 column.
4   The tryptic peptides containing the phosphorylated
5   residues Ser-124, Thr-308, Thr-450, Ser-473 are marked
6   and their assignments were confirmed by phosphoamino
7   acid analysis and sequencing to identify the sites of
8   phosphorylation (data not shown). The phosphopeptides
9   containing Thr-308 and Ser-473 were absent if
10 stimulation with IGF-1 was omitted, while the
11 phosphopeptides containing Ser-124 and Thr-450 were
12 present at similar levels as observed with wild-type
13 PKBα (see Fig 9A). Similar results were obtained in 3
14 separate experiments.
15
16 These experiments demonstrated that IGF-1 stimulation
17 induced the phosphorylation of HA-473A PKBα at Thr-308,
18 and the phosphorylation of HA-308A PKBα at Ser-473.
19 Similar results were obtained after stimulation with
20 insulin rather than IGF-I.
21
22 Example 7: IGF-1 or insulin induces phosphorylation of
23 Thr-308 and Ser-473 in a catalytically inactive mutant
24 of PKBα.
25
26 Fig 15. The catalytically inactive PKBα mutant
27 (HA-KD-PKBα) expressed in 293 cells is phosphorylated
28 at Thr-308 and Ser-473 after stimulation with IGF-1.
29 Each 10 cm dish of 293 cells transiently transfected
30 with HA-KD-PKBα DNA constructs was ^{32}P-labelled and
31 incubated for 10 min with buffer (A), 50 ng/ml IGF-1
32 (B) or 100 nM insulin (C). The ^{32}P-labelled HA-KD-PKBα
33 was immunoprecipitated from the lysates, treated with 4
34 vinylpyridine, electrophoresed on a 10% polyacrylamide
35 gel, excised from the gel and digested with trypsin,
36 then chromatographed on a C18 column. The tryptic

1   peptides containing the phosphorylated residues
2   Ser-124, Thr-308, Thr-450 and Ser-473 are marked.
3   Similar results were obtained in 3 separate experiments
4   for (A) and (B), and in two experiments for (C).
5
6   This kinase dead" mutant of PKBα, termed HA-KD-PKBα, in
7   which Lys-179 was changed to Ala (see above) was
8   transiently expressed in 293 cells and its level of
9   expression found to be several-fold lower than that of
10   wild type HA-PKBα expressed under identical conditions
11   (Fig 8B). As expected, no PKBα activity was detected
12   when 293 cells expressing HA-KD-PKBα, were stimulated
13   with insulin or IGF-1 (Fig 7A).
14
15   293 cells that had been transiently transfected with
16   HA-KD-PKBα were ^{32}P-labelled, then stimulated with
17   buffer, insulin or IGF-1. and sites on PKBα
18   phosphorylated under these conditions were mapped. In
19   contrast to wild type HA-PKBα from unstimulated 293
20   cells (Fig 9), HA-KD PKBα was phosphorylated to a much
21   lower level at Ser-124, but phosphorylated similarly at
22   Thr-450 (Fig 15A). Following stimulation with IGF-1
23   (Fig 15B) or insulin (Fig 14C) HA-KD-PKBα became
24   phosphorylated at the peptides containing Thr-308 and
25   Ser-473, the extent of phosphorylation of these sites
26   being at least as high as wild type PKBα. Amino acid
27   sequencing of these peptides established that they were
28   phosphorylated at Thr-308 and Ser-473, respectively.
29
30   The above examples establish that PKB influences GSK3
31   activity; that Thr-308 and Ser-473 are the major
32   residues in PKBα that become phosphorylated in response
33   to insulin or IGF-1 (Figs 2 and 5) and that
34   phosphorylation of both residues is required to
35   generate a high level of PKBα activity. Thus mutation
36   of either Thr-308 or Ser-473 to Ala greatly decreased

1   the activation of transfected PKBα by insulin or IGF-1
2   in 293 cells (Fig 8). Moreover, PKBα became partially
3   active in vitro when either Thr-308 or Ser-473 were
4   changed to Asp or when Ser-473 was phosphorylated by
5   MAPKAP kinase-2 in vitro, and far more active when the
6   D308 mutant of PKBα was phosphorylated by MAPKAP
7   kinase-2 or when Thr-308 and Ser-473 were both mutated
8   to Asp (Fig 12). Moreover, the D308/D473 double mutant
9   could not be activated further by stimulating cells
10   with insulin (Fig 13). These observations demonstrate
11   that the phosphorylation of Thr-308 and Ser-473 act
12   synergistically to generate a high level of PKBα
13   activity.
14
15   Thr-308, and the amino acid sequence surrounding it, is
16   conserved in rat PKBβ and PKBγ but, interestingly,
17   Ser-473 (and the sequence surrounding it) is only
18   conserved in PKBβ. In rat PKBγ, Ser-473 is missing
19   because the C-terminal 23 residues are deleted. This
20   suggests that the regulation of PKBγ may differ
21   significantly from that of PKBα and PKBβ in the rat.
22
23   Thr-308 is located in subdomain VIII of the kinase
24   catalytic domain, nine residues upstream of the
25   conserved Ala-Pro-Glu motif, the same position as
26   activating phosphorylation sites found in many other
27   protein kinases. However, Ser-473 is located C-terminal
28   to the catalytic domain in the consensus sequence
29   Phe-Xaa-Xaa-Phe/Tyr-Ser/Thr-Phe/Tyr which is present in
30   several protein kinases that participate in growth
31   factor-stimulated kinase cascades, such as p70 S6
32   kinase, PKC and p90rsk (Pearson et al, 1995). However,
33   it is unlikely that a common protein kinase
34   phosphorylates this motif in every enzyme for the
35   following reasons. Firstly, phosphorylation of the
36   equivalent site in p70 S6 kinase is prevented by the

1   immunosuppressant drug rapamycin (Pearson et al, 1995)
2   which does not prevent the activation of PKBα by
3   insulin (Cross et al, 1995) or is phosphorylation at
4   Ser-473 (D. Alessi, unpublished work). Secondly, the
5   equivalent residue in protein kinase C is
6   phosphorylated constitutively and not triggered by
7   stimulation with growth factors (Tsutakawa et al.,
8   1995).
9
10   MAPKAP kinase-2 is a component of a protein kinase
11   cascade which becomes activated when cells are
12   stimulated with interleukin-1 or tumour necrosis factor
13   or exposed cellular stresses (Rouse et al, 1994; Cuenda
14   et al, 1995). MAPKAP kinase-2 phosphorylates PKBα
15   stoichiometrically at Ser-473 (Fig 11) and this finding
16   was useful in establishing the role of Ser473
17   phosphorylation in regulating PKBα activity. However,
18   although MAPKAP kinase-2 activity is stimulated to a
19   small extent by insulin in L6 cells, no activation
20   could be detected in 293 cells in response to insulin
21   or IGF-1. Moreover, exposure of L6 cells or 293 cells
22   to a chemical stress (0.5 mM sodium arsenite) strongly
23   activated MAPKAP kinase-2 (D. Alessi, unpublished work)
24   as found in other cells (Rouse et al, 1994; Cuenda et
25   al, 1995), but did not activate PKBα at all.
26   Furthermore, the drug SB 203580 which is a specific
27   inhibitor of the protein kinase positioned immediately
28   upstream of MAPKAP kinase-2 (Cuenda et al, 1995),
29   prevented the activation of MAPKAP kinase-2 by arsenite
30   but had no effect on the activation of PKBα by insulin
31   or IGF-1. Finally, the activation of PKBα was prevented
32   by wortmannin (Figs 6 and 9), but wortmannin had no
33   effect on the activation of MAPKAP kinase-2 in L6 or
34   293 cells. It should also be noted that the sequence
35   surrounding Ser-473 of PKBα (HFPQFSY) does not conform
36   to the optimal consensus for phosphorylation by MAPKAP

kinase-2 which requires Arg at position n-3 and a bulky
hydrophobic residue at position n-5, (where n is the
position of the phosphorylated residue). The Km for
phosphorylation of the synthetic peptide comprising
residues 465-478 is nearly 100-fold higher than the Km
for the standard MAPKAP kinase-2 substrate peptide
(data not shown). It is therefore unlikely that MAPKAP
kinase-2 mediates the phosphorylation of Ser-473 in
vivo.

The enzyme(s) which phosphorylates Thr-308 and Ser-473
in vivo does not appear to be PKBα itself. Thus
incubation of the partially active Asp-308 mutant with
MgATP did not result in the phosphorylation of Ser-473,
phosphorylation of the latter residue only occurring
when MAPKAP kinase-2 was added (Fig 11A, Fig 12).
Similarly, Thr-308 did not become phosphorylated when
either the partially active D473 mutant or the
partially active Ser-473 phosphorylated form of PKBα
were incubated with MgATP. PKBα when bound to lipid
vesicles containing phosphatidylserine and PIP3 also
fails to activate upon incubation with MgATP (Alessi et
al, 1996) and after transfection into 293 cells, a
"kinase dead" mutant of PKBα became phosphorylated on
Thr-308 and Ser-473 in response to insulin or IGF-1
(Fig 14). Furthermore, HA-PKBα from either unstimulated
or insulin-stimulated 293 cells failed to phosphorylate
the synthetic C-terminal peptide comprising amino acids
467-480.

In unstimulated L6 myotubes, the endogenous PKBα was
phosphorylated at a low level at a number of sites (Fig
6A), but in unstimulated 293 cells the transfected
enzyme was heavily phosphorylated at Ser-124 and
Thr-450 (Fig 10). Ser-124 and Thr-450 are both followed
by proline residues suggesting the involvement of

1   "proline-directed" protein kinases. Although, the
2   phosphorylation of Ser-124 was greatly decreased when
3   "kinase dead" PKBα was transfected into 293 cells (Fig
4   14), it would be surprising if Ser-124 is
5   phosphorylated by PKBα itself because the presence of a
6   C-terminal proline abolishes the phosphorylation of
7   synthetic peptides by PKBα (D.Alessi, unpublished
8   work). Since transfected PKBα is inactive in
9   unstimulated 293 cells (Fig 12), phosphorylation of
10   Ser-124 and Thr-450 clearly does not activate PKBα
11   directly. Ser-124 is located in the linker region
12   between the PH domain and the catalytic domain of the
13   mammalian PKBα isoforms but, unlike Thr-450, is not
14   conserved in the Drosophila homologue (Andjelkovic et
15   al, 1995).
16
17   While we do not wish to be bound by hypotheses, the
18   results described suggest that agonists which activate
19   PI 3-kinase are likely to stimulate PKBα activity via
20   one of the following mechanisms. Firstly, PIP3 or
21   PI3,4-bisP may activate one or more protein kinases
22   which then phosphorylate PKBα at Thr-308 and Ser-473.
23   Secondly, the formation of PIP3 may lead to the
24   recruitment of PKBα to the plasma membrane where it is
25   activated by a membrane-associated protein kinase(s).
26   The membrane associated Thr-308 and Ser-473 kinases
27   might themselves be activated by PIP3 and the
28   possibility that Thr-308 and/or Ser-473 are
29   phosphorylated directly by PI 3-kinase has also not
30   been excluded, because this enzyme is known to
31   phosphorylate itself (Dhand et al, 1994) and other
32   proteins (Lam et al, 1994) on serine residues.
33
34   Example 8: Molecular basis for substrate specificity of
35   PKB. PKBα has been shown to influence GSK3 activity.
36   GSK3α and GSK3β are phosphorylated at Ser-21 and Ser-9,

1 respectively, by two other insulin-stimulated protein
2 kinases, namely p70 S6 kinase and MAP kinase-activated
3 protein kinase-1 (MAPKAP-K1, also known as p90 S6
4 kinase). However, these enzymes are not rate-limiting
5 for the inhibition of GSK3 by insulin in L6 myotubes
6 because specific inhibitors of their activation
7 (rapamycin-p70 S6 kinase; PD 98059-MAPKAP kinase-1)
8 have no effect (Cross et al., 1995).
9
10 The activation of PI 3-kinase is essential for many of
11 the effects of insulin and growth factors, including
12 the stimulation of glucose transport, fatty acid
13 synthesis and DNA synthesis, protection of cells
14 against apoptosis and actin cytoskeletal rearrangements
15 (reviewed in Carpenter et al., 1996). These
16 observations raise the question of whether PKBα
17 mediates any of these events by phosphorylating other
18 proteins. To address this issue we characterised the
19 substrate specificity requirements of PKBα. We find
20 that the optimal consensus sequence for phosphorylation
21 by PKBα is the motif Arg-Xaa-Arg-Yaa-Zaa-Ser/Thr-Hyd,
22 where Yaa and Zaa are small amino acids (other than
23 glycine) and Hyd is a large hydrophobic residue (such
24 as Phe or Leu). We also demonstrate that PKBα
25 phosphorylates histone H2B (a substrate frequently used
26 to assay PKBα in vitro) at Ser-36 which lies in an Arg-
27 Xaa-Arg-Xaa-Xaa-Ser-Hyd motif. These studies identified
28 a further PKBα substrate (Arg-Pro-Arg-Ala-Ala-Thr-Phe)
29 that, unlike other peptides, is not phosphorylated to a
30 significant extent by either p70 S6 kinase or MAPKAP-
31 K1.
32
33
34 Results
35 Preparation of Protein Kinase Bα
36 In order to examine the substrate specificity of PKBα,

1   it was first necessary to obtain a kinase preparation
2   that was not contaminated with any other protein kinase
3   activities.   293 cells were therefore transiently
4   transfected with a DNA construct expressing
5   haemagglutonin-tagged PKBα (HA-PKBα), stimulated with
6   IGF-1 and the HA-PKBα immunoprecipitated from the
7   lysates). IGF-1 stimulation resulted in a 38-fold
8   activation of PKBα (Fig 16) and analysis of the
9   immunoprecipitates by SDS-polyacrylamide gel
10   electrophoresis revealed that the 60 kDa PKBα was the
11   major protein staining with Coomassie Blue apart from
12   the heavy and light chains of the haemagglutonin
13   monoclonal antibody (Fig 16, Lanes 2 and 3). The minor
14   contaminants were present in control immunoprecipitates
15   derived from 293 cells transfected with an empty pCMV5
16   vector but lacked HA-PKB activity (Fig 16, Lane 4).
17   Furthermore, a catalytically inactive mutant HA-
18   PKBα_immunoprecipitated from the lysates of IGF-1
19   stimulated 293 cells had no Crosstide kinase activity
20   (Alessi et al., 1996). Thus, all the Crosstide activity
21   in HA-PKB immunoprecipitates is catalysed by PKBα.
22
23   **Identification of the residues in histone H2B**
24   **phosphorylated by PKBα.** Currently, three substrates are
25   used to assay PKBα activity in different laboratories,
26   histone H2B, MBP and Crosstide. PKBα phosphorylated
27   Crosstide with a Km of 4 μM and a Vmax of 260 U/mg
28   (Table 7.1 A, peptide 1), histone H2B with a Km of 5 μM
29   and a Vmax of 68 U/mg, and MBP with a Km of 5 μM and a
30   Vmax of 25 U/mg. Thus the Vmax of histone H2B and MBP
31   are 4-fold and 10-fold lower than for Crosstide. In
32   order to identify the residue(s) in histone H2B
33   phosphorylated by PKBα, ^{32}P-labelled histone H2B was
34   digested with trypsin (see Methods) and the resulting
35   peptides chromatographed on a C18 column at pH 1.9.
36   Only one major ^{32}P-labelled peptide (termed T1) eluting

at 19.5 % acetonitrile was observed (Fig 17A), The peptide contained phosphoserine (data not shown), its sequence commenced at residue 34 of histone H2B and a single burst of radioactivity occurred after the third cycle of Edman degradation (Fig 17B), demonstrating that PKBα phosphorylates histone H2B at Ser-36 within the sequence Arg-Ser-Arg-Lys-Glu-Ser-Tyr. Thus, like the serine phosphorylated in Crosstide, Ser-36 of histone H2B lies in an Arg-Xaa-Arg-Xaa-Xaa-Ser-Hyd motif (where Hyd is a bulky hydrophobic residue -Phe in Crosstide, Tyr in H2B).

Molecular basis for the substrate specificity of PKBα

To further characterise the substrate specificity requirements for PKBα, we first determined the minimum sequence phosphorylated efficiently by PKBα by removing residues sequently from the C-terminal and N-terminal end of Crosstide. Removal of the N-terminal glycine and up to three residues from the C-terminus had little effect on the kinetics of phosphorylation by PKBα (Table 7.1A, compare peptides 1 and 5). However any further truncation of either the N or C-terminus virtually abolished phosphorylation (Table 7.1A, peptides 8 and 9). The minimum peptide phosphorylated efficiently by PKBα (Arg-Pro-Arg-Thr-Ser-Ser-Phe) was found to be phosphorylated exclusively at the second serine residue as expected. Consistent with this finding, a peptide in which this serine was changed to alanine was not phosphorylated by PKBα (Table 7.1A, peptide 7). All further studies were therefore carried out using variants of peptide 5 in Table 7.1A (see below).

A peptide in which the second serine of peptide 5 (Table 7.1A) was replaced by threonine was phosphorylated with a K_m of 30 μM and an unchanged Vmax

1   (Table 7.1, peptide 6). All the ^{32}P-radioactivity
2   incorporated was present as phosphothreonine and solid
3   phase sequencing revealed that the peptide was only
4   phosphorylated at the second threonine residue, as
5   expected (data not shown). Thus PKBα is capable of
6   phosphorylating threonine as well as serine residues,
7   but has a preference for serine.
8
9   We next changed either of the two arginine residues in
10   peptide 5 to lysine. These substitutions drastically
11   decreased the rate of phosphorylation by PKBα (Table
12   7.1A, peptides 10 and 11) demonstrating a requirement
13   for arginine (and not simply any basic residue) at both
14   positions.
15
16   We then examined the effect of substituting the
17   residues situated immediately C-terminal to the
18   phosphorylated serine in peptide 5 (Table 7.1B). The
19   data clearly demonstrate that the presence of a large
20   hydrophobic residue at this position is critical for
21   efficient phosphorylation, with the Km increasing
22   progressively with decreasing hydrophobicity of the
23   residue at this position (Table 7.1B, peptides 1 to 4).
24   Replacement of the C-terminal residue with Lys
25   increased the Km 18-fold and a substitution at this
26   position with either Glu or·Pro almost abolished
27   phosphorylation (Table 7.1B, peptides 5-7).
28
29   Replacement of the Thr situated two residues N-terminal
30   to the phosphorylated serine increased the Km with any
31   amino acid tested (Table 7.1C). Substitution with Ala
32   only increased Km by 2-3 fold, but substitution with
33   other residues was more deleterious and with Asn (a
34   residue of similar size and hydrophilicity to Thr)
35   phosphorylation was almost abolished (Table 7.1C).
36   Replacement of the Ser situated one residue N-terminal

1   to the phosphorylated serine also increased the Km with

2   any amino acid tested, but the effects were less severe

3   than at position n-2 (Table 7.1C). When residues n-2

4   and n-1 were both changed to Ala, the resulting peptide

5   RPRAASF was phosphorylated by PKBα with a Km only 5-

6   fold higher than RPRTSSF. In contrast the peptides

7   RPRGGSF, RPRAGSF, and RPRGASF were phosphorylated less

8   efficiently (Table 7.1C).

9

10   **Comparison of the substrate specificity of PKBα with**

11   **MAPKAP kinase-1, and p70 S6 kinase.** Since MAPKAP-K1

12   and p70 S6 kinase phosphorylate the same residue in

13   GSK3 phosphorylated by PKBα, and studies with synthetic

14   peptides have established that MAPKAP-K1 and p70 S6

15   kinase also preferentially phosphorylate peptides in

16   which basic residues are present at positions n-3 and

17   n-5 (Leighton et al., 1995), we compared the

18   specificities of MAPKAP-K1, p70 S6 kinase and PKBα in

19   greater detail.

20

21   MAPKAP kinase-1 and p70 S6 kinase phosphorylate the

22   peptides KKKNRTLSVA and KKRNRTLSVA with extremely low

23   Km values of 0.2- 3.3 μM, respectively (Table 7.2).

24   However, these peptides were phosphorylated by PKBα

25   with 50-900 fold higher Km values (Table 7.2A, peptides

26   1 and 2). The peptide KKRNRTLTV, which is a relatively

27   specific substrate for p70 S6 kinase (Leighton et al.,

28   1995) was also phosphorylated very poorly by PKBα

29   (Table 7.2A, peptide 4).

30

31   Crosstide is phosphorylated by p70 S6 kinase and MAPKAP

32   kinase-1 with similar efficiency to PKBα ((Leighton et

33   al., 1995); Table 7.2B-peptide 1 and Fig 18). However,

34   truncation of Crosstide to generate the peptide RPRTSSF

35   was deleterious for phosphorylation by MAPKAP-K1 and

36   even worse for p70 S6 kinase (Table 7.2B-peptides 1 and

1   2 and Fig 18). Moreover, changing the phosphorylated
2   serine in RPRTSSF to threonine increased the Km for
3   phosphorylation by p70 S6 kinase much more than for
4   PKBα and almost abolished phosphorylation by MAPKAP-K1
5   (Table 7.2B-peptide 3 and Fig 18). The peptide RPRAASF
6   was phosphorylated by MAPKAP-K1 with essentially
7   identical kinetics to that of PKBα; however
8   phosphorylation by p70 S6 kinase was virtually
9   abolished (Table 7.2B-peptide 4 and Fig 18). Based on
10 these observations we synthesized the peptide RPRAATF.
11 This peptide was phosphorylated by PKBα with a Km of
12 25μM and similar Vmax to RPRTSSF, but was not
13 phosphorylated to a significant extent by either
14 MAPKAP-K1 or p70 S6 kinase (Table 7.2B-peptide 5, Fig
15 18). In Fig 18, the protein kinase concentration in
16 the assays towards Crosstide were 0.2 U/ml, and each
17 peptide substrate was assayed at a concentration of 30
18 μM. Filled bars denote PKBα activity, hatched bars
19 MAPKAP kinase-1 activity, and grey bars p70 S6 kinase
20 activity. The activities of each protein kinase are
21 given relative to their activity towards Crosstide
22 (100). The results are shown ± SEM for two experiments
23 each carried out in triplicate.
24
25 ### Discussion.
26 We have established that the minimum consensus sequence
27 for efficient phosphorylation by PKBα is Arg-Xaa-Arg-
28 Yaa-Zaa-Ser-Hyd, where Xaa is any amino acid, Yaa and
29 Zaa are small amino acid other than glycine (Ser, Thr,
30 Ala) and Hyd is a bulky hydrophobic residue (Phe, Leu)
31 (Table 7.1). The heptapeptide with the lowest Km value
32 was RPRTSSF, its Km of 5 μM being comparable to many of
33 the best peptide substrates identified for other
34 protein kinases. The Vmax for this peptide (250 nmoles
35 min-1 mg-1) may be an underestimate because the PKBα
36 was obtained by immunoprecipitation from extracts of

IGF-1 stimulated 293 cells over-expressing this protein
kinase, and a significant proportion of the PKBα may
not have been activated by IGF-1 treatment.

The requirement for arginine residues at positions n-3
and n-5 (where n is the site of phosphorylation) seems
important, because substituting either residue with
lysine decreases phosphorylation drastically. Serine
and threonine residues were preferred at positions n-1
and n-2, although the Km value was only increased about
5-fold if both of these residues were changed to Ala.
Serine was preferred at position n, since changing it
to threonine caused a six-fold increase in the Km.
The peptide RPRAATF, which was phosphorylated with a Km
of 25 μM and similar Vmax to RPRTSSF, may therefore be
a better substrate for assaying PKBα in partially
purified preparations, because unlike Crosstide, it
contains only one phosphorylatable residue and is not
phosphorylated significantly by MAPKAP-K1 or p70 S6
kinase (Table 7.2, Fig 18 and see below).

The Proline at position n-4 was not altered in this
study because it was already clear that this residue
was not critical for the specificity of PKBα. Residue
n-4 is proline in GSK3β but alanine in GSK3α. Both GSK3
isoforms are equally good substrates for PKBα in vitro
(Cross et al., 1995), and the peptide
GRARTSSFA (corresponding to the sequence in GSK3α) is
phosphorylated by PKBα with a Km of 10 μM and Vmax of
230 U/mg (Table 7.1A, peptide 2). Moreover, in histone
H2B, the residue located four amino acids N-terminal to
the PKBα phosphorylation site is serine (Fig 17). The
presence of Glu and Lys at positions n-1 and n-2 may
explain why histone H2B is phosphorylated by PKBα with
a four-fold lower Vmax than the peptide RPRTSSF.

1   Two other protein kinases which are activated by

2   insulin and other growth factors, p70 S6 kinase and

3   MAPKAP-K1, require basic residues at positions n-3 and

4   n-5 (Leighton et al., 1995), explaining why they also

5   phosphorylate and inactivate GSK3 in vitro (Sutherland

6   et al., 1993). Indeed, there is evidence that MAPKAP-

7   K1 plays a role in the inhibition of GSK3 by EGF

8   because, unlike inhibition by insulin which is

9   prevented by inhibitors of PI 3-kinase, the inhibition

10   of GSK3 by EGF is only suppressed partially by

11   inhibitors of PI 3-kinase. Moreover, in NIH 3T3 cells,

12   the inhibition of GSK3α and GSK3β by EGF is largely

13   prevented by expression of a dominant negative mutant

14   of MAP kinase kinase-1 (Eldar et al., 1995). In

15   contrast, p70 S6 kinase is not rate limiting for the

16   inhibition of GSK3 in the cells that have been examined

17   so far because rapamycin, which prevents the activation

18   of p70 S6 kinase by EGF or insulin, has no effect on

19   the inhibition of GSK3 by these agonists (Cross et al.,

20   1995 and Saito et al., 1994).

21

22   Additional similarities between p70 S6 kinase, MAPKAP-

23   K1 and PKBα include the failure to phosphorylate

24   peptides containing Pro at position n+1 and dislike of

25   a lysine at the same position. This suggests that, in

26   vivo, these kinases are unlikely to phosphorylate the

27   same residues as MAP kinases (which phosphorylates

28   Ser/Thr-Pro motifs) or protein kinase C (which prefers

29   basic residues C-terminal to the site of

30   phosphorylation). However, the present work has also

31   revealed significant differences in the specificities

32   of these enzymes. In particular MAPKAP-K1 and (to a

33   lesser extent) p70 S6 kinase can tolerate substitution

34   of the Arg at position n-5 by lysine whereas PKBα

35   cannot (see Table 7.1A, Table 7.2A and (Leighton et

36   al., 1995)). MAPKAP-K1 and p70 S6 kinase can also

1 tolerate, to some extent, substitution of Arg at
2 position n-3 by Lys.   For example, the peptide
3 KKRNKTLSVA is phosphorylated by MAPKAP-K1 and p70 S6
4 kinase with Km values of 17 and 34 μM, respectively,
5 as compared to Km values of 0.7 and 1.5 μM for the
6 peptide ·KKRNRTLSVA (Table 7.2A).   In contrast, PKBα
7 does not phosphorylate the peptide KKRNKTLSVA (Table
8 7.2A) or any other peptide that lacks Arg at position
9 n-3. PKBα and p70 S6 kinase, but not MAPKAP-K1,
10 phosphorylate Thr as well as Ser (Table 7.1A) and can
11 phosphorylate peptides lacking any residue at position
12 n+2 ((Leighton et al., 1995) and Table 7.2A), while
13 PKBα and MAPKAP-K1, but not p70 S6 kinase, can tolerate
14 substitution of both the n-1 and n-2 positions of the
15 peptide RPRTSSF with Ala (Table 7.2B).   These
16 differences explain why the peptide RPRAATF is a
17 relatively specific substrate for PKBα.
18
19 One of the best peptide substrates for MAPKAP-K1 and
20 p70 S6 kinase (KKRNRTLSVA) was a poor substrate for
21 PKBα (Table 7.2, peptide 2), despite the presence of
22 Arg at positions n-3 and n-5. The presence of Leu at
23 position n-1 and Val at position n+1 are likely to
24 explain the high Km for phosphorylation, because PKBα
25 prefers a small hydrophilic residue at the former
26 position and a larger hydrophobic residue at the latter
27 position (Tables 7.1 and 7.2).
28
29 **Example 9:**
30 This example demonstrates that coexpression of GSK3 in
31 293 cells with either the wild type or a constitutively
32 activated PKB results in GSK3 becoming phosphorylated
33 and inactivated. However coexpression of a mutant of
34 GSK3 in which Ser-9 is mutated to an Ala residue is not
35 inactivated under these conditions. These experiments
36 provide further evidence that PKBa activation can

1   mediate the phosphorylation and inactivation of GSK3 in

2   a cellular environment, and could be used as an assay

3   system to search for specific PKB inhibitors.

4

5   Monoclonal antibodies recognising the sequence EFMPME

6   (EE) antibodies and the (EQKLISEEDL) c-Myc purchased

7   from Boehringer (Lewis, UK).

8

9   **Construction of expression vectors and transfections**

10   **into 293 cells.** HA-PKBa, HA-KD-PKB and 308D/473D

11   HA-PKBa was described previously (Alessi et al.. 1996).

12

13   A DNA construct expressing human GSK3B with the EFMPME

14   (EE) epitope tag at the N-terminus was prepared as

15   follows: A standard PCR reaction was carried out using

16   as a template the human GSK3β cDNA clone in the

17   pBluescript SK+ vector and the oligonucleotides

18

19   GCGG<u>AGATCT</u>GCCACCATGGAGTTCATGCCCATGGAGTCAGGGCGGCCCAGAACC

20

21   and GCGG<u>TCCGGA</u>ACATAGTCCAGCACCAG that incorporate a *bgl*

22   II site (underlined) and a *Bspe I* site (double

23   underlined). A three-way ligation was then set up in

24   which the resulting PCR product was subcloned as a *Bgl*

25   *II-Bspe* I fragment together with the C-terminal Bspe

26   I-Cla I fragment of GSK3β into the Bgl II-Cla I sites

27   of the pCMV5 vector (Anderson et al., 1989). The

28   construct was verified by DNA sequencing and purified

29   using the Quiagen plasmid Mega kit according to the

30   manufacturers protocol. The c-Myc GSK3, BA9 construct

31   encodes GSK3β in which Ser-9 is mutated to Ala and

32   possesses a c-myc epitope tag at the C-terminus and was

33   prepared as described in Sperber et al., 1995. The

34   c-Myc GSK3β A9 gene was then subcloned into xba I/ECOR

35   I sites of the pCMV5 eukaryotic expression vector.

36

1   **Cotransfection of GSK3β with PKBa and its assay.**
2   293 cells growing on 10 cm diameter dishes were
3   transfected with 10 ug of DNA constructs expressing
4   EE-GSK3, Myc-GSK3A9 in the presence or absence of
5   HA-PKB, HA-KD-PKB or HA-308D/473D-PKB exactly as
6   described in Alessi et al., 1996. The cells were grown
7   in the absence of serum for 16 h prior to lysis, and
8   then lysed in 1.0 ml of ice-cold Buffer A (50 mM
9   Tris/HCI pH 7.5,1 mM EDTA 1 mM EGTA, 1% (by vol) Triton
10   X100, 1 mM sodium orthopervanadate, 10 mM sodium
11   glycerophosphate, 50 mM NaF, 5 mM sodium pyrophosphate,
12   1uM Microcystin-LR, 0.27 M sucrose, 1 mM benzamidine,
13   0.2 mM phenylmethylsulphonyl fluoride, 10 ug/ml
14   leupeptin, and 0.1% (by vol) 2-mercaptoethanol). The
15   lysate was centrifuged at 4°C for 10 min at 13, 000 x g
16   and an aliquot of the supernatant (100 ug protein) was
17   incubated for 30 min on a shaking platform with 5 ul of
18   protein G-Sepharose coupled to 1ug of EE monoclonal
19   antibody. The suspension was centrifuged for 1min at
20   13,000 x g, the Protein G-Sepharose-antibody-EE-GSK3β
21   complex washed twice with 1.0 ml of Buffer A containing
22   0.5 M NaCl, and three times with Buffer B (50 mM Tris
23   pH 7.5, 0.1 mM EGTA, 0.01% (by vol) Brij-35 and 0.1%
24   (by vol) 2-mercaptoethanol), and the immunoprecipitate
25   assayed for GSK3 activity after incubation with either
26   PP2A or microcystin inactivated PP2A as described
27   previously (Cross et al., 1994).
28
29   **Results**
30
31   **Cotransfection of GSK3β with PKBa in 293 cells results**
32   **in GSK3 phosphorylation and inactivation**
33   Human embryonic kidney 293 cells were transfected with
34   a DNA construct expressing EE-epitope tagged GSK3β
35   either in the presence or absence of DNA constructs
36   expressing wild type-PKBa, a catalytically inactive

1   PKBa or a constitutively active HA-(308D/473D)-PKBa.

2   Cells were serum starved for 16 h.   36h post

3   transfection the cells were lysed, and the GSK3β

4   immunoprecipitated from the lysates using monoclonal EE

5   antibodies and the GSK3β activity measured before and

6   after treatment with PP2A. When EEGSK3β was expressed

7   alone or in the presence of a catalytically inactive

8   PKBa, treatment of the EE-GSK3β with PP2A only resulted

9   in about a 12% increase in activity (Fig 19A). However

10 when EE-GSK3β was coexpressed with either the wild type

11 PKBa or the constitutively activated 308D/473D-HA-PKBa,

12 treatment of the EE-GSK3 from these cell lysates with

13 PP2A resulted in a 68% and 85% increase in the GSK3

14 activity, respectively. Coexpression of Myc-GSK3β A9

15 with HA-PKB or the constitutively active

16 308D/473D-HA-PKBa did not result in any significant

17 inactivation of this mutant of GSK3 as judged by its

18 ability to be reactivated by PP2A (Fig 19B). These data

19 demonstrate that even in a cellular environment, PKBa

20 is capable of phosphorylating GSK3β at Ser-9 and

21 inactivation of the enzyme. To estimate the relative

22 levels of EE-GSK3β and PKBa, EE-GSK3 and HA-PKBa were

23 immunoprecipitated from equal volumes of cell lysate,

24 and the immunoprecipitates run on an SDS-polyacrylamide

25 gel, and the gel stained with Coomassie Blue. These

26 experiments revealed that both the wild type HA-PKBa

27 and the 308D/473D-PKBa were expressed at a 20 to 30

28 -fold higher level than GSK3a, whereas KD-PKBa is

29 expressed at a level that is about 5-fold lower than

30 that of the wild type PKBa. Under the conditions used

31 for the immunoprecipitations, no PKBa was

32 co-immnuoprecipitated with GSK3β, or no GSK3β was

33 co-immunoprecipitated with the PKBa (data not shown).

34 Coexpression of EE-GSK3β with all forms of PKBa

35 resulted in about a 2-3 fold decrease in the level of

36 expression on EE-GSK3β compared to when it is expressed

1   alone in cells.

2

3   **Example 10: basic assay for identifying agents which**

4   **affect the activity of PKB.**

5   A 40 μl assay mix was prepared containing protein

6   kinase (0.2U/ml) in 50 mM Tris/HCI pH 7.5, 0.1 mM EGTA,

7   0.1% (by vol) 2-mercaptoethanol, 2.5 μM PKI, protein

8   kinase substrate (30μM), and the indicated

9   concentration of Ro-318220 or GC 109203X (test

10   inhibitors). After incubation on ice for 10 min the

11   reaction was started by the addition of 10 μl of 50mM

12   magnesium acetate and 0.5 mM [γ^{32}P]ATP (100-200

13   cpm/pmol). For the assay of mixed isoforms of PKC 20

14   μM diacylglycerol, 0.5 mM CaCl$_2$, and 100 μM

15   phosphatidylserine were also present in the

16   incubations. The assays were carried out for 15 min at

17   30°C, then terminated and analysed as described (Alessi

18   1995). One unit of activity was that amount of enzyme

19   that catalysed the phosphorylation of 1nmol os

20   substrate in 1 min. The final concentration of DMSo in

21   each assay was 1% (by vol). This concentration of DMSO

22   does not inhibit any of these enzymes. Mixed isoforms

23   of PKC were assayed using histone H1 as substrate,

24   while MAPKAP-K1β and p70 S6 kinase were assayed using

25   the peptide KKRNRTLSVA (Leighton 1995). Protein kinase

26   B was assayed with the peptide GRPRTSSFAEG [9] and

27   MAPKAP-K2 was assayed with the peptide KKLNRTLSVA

28   (Stokoe 1993). p42 MAP kinase was assayed using MBP,

29   and MAPKK-1, and c-Raf1 were assayed as described in

30   Alessi 1995.

31

32   **Results**

33   Effect of Ro 318220 and GF 109203X on protein kinases

34   activated by growth factors, cytokines and cellular

35   stresses. The mixed isoforms of PKC were potently

36   inhibited by Ro 318220, with an IC$_{50}$ of 5 nM in our

1 assay (Fig 20A). In contrast, a number of protein
2 kinases activated by growth factors (c-Raf1, MAPKK-1,
3 p42 MAP kinase) and one protein kinase that is
4 activated by cellular stresses and proinflammatory
5 cytokines (MAPKAP-K2) were not inhibited significantly
6 by Ro 318022 in vitro (Fig 20A). Protein kinase B, an
7 enzyme that is activated in response to insulin and
8 growth factors was inhibited by Ro 318220 (IC_{50} of 1 μM,
9 Fig 20B) similar to the IC_{50} for PKA. However, to our
10 surprise, MAPKAP-K1B an enzyme which lies immediately
11 downstream of p42 and p44 MAP kinases and which is
12 activated in response to every agonist that stimulates
13 this pathway, was inhibited by Ro 318220 even more
14 potently than the mixed PKC isoforms (IC_{50} = 3nm, Fig
15 20B). The p70 S6 kinase, which lies on a distinct
16 growth factor-stimulated signalling pathway from
17 MAPKAP-K1B, was also potently inhibited by Ro 318220
18 (IC_{50}=15 nM, Fig 20B).
19
20 Similar results were obtained using GF 109203X instead
21 of Ro 3318220. As reported previously (Toullec et al.,
22 1991), GC 109203X inhibited the mixed isoforms of PKC
23 (IC_{50}=30 nM) without inhibiting protein kinase B (Fig
24 21) or c-Raf, MAPKK-1 and p42 MAP kinase (data not
25 shown). However MAPKAP-K1B and p70 S6 kinase were
26 potently inhibited by this compound with IC_{50} values of
27 50 nM and 100 nM, respectively (Fig 21).
28
29

1   **General Materials and Methods** Tissue culture reagents, myelin
2   basic protein (MBP), microcystin-LR, and IGF-1 were obtained from
3   Life Technologies Inc. (Paisley, UK), insulin from Novo-Nordisk
4   (Bagsvaerd, Denmark), phosphate free Dulbecco's minimal essential
5   medium (DMEM) from (ICN, Oxon, UK), Protein G-Sepharose and
6   CH-Sepharose from Pharmacia (Milton Keynes, UK), alkylated trypsin
7   from Promega (Southampton, UK), 4-vinylpyridine, wortmannin and
8   fluroisothiocyanante-labelled antimouse IgG from goat from
9   Sigma-Aldrich (Poole, Dorset, UK). Polyclonal antibodies were
10   raised in sheep against the peptides RPHFPQFSYSASGTA
11   (corresponding to the last 15 residues of rodent PKBα) and
12   MTSALATMRVDYEQIK (corresponding to residues 352 to 367 of human
13   MAPKAPkinase-2) and affinity purified on peptide-CH-Sepharose.
14   Monoclonal HA antibodies were purified from the tissue culture
15   medium of 12CA5 hybridoma and purified by chromatography on
16   Protein GSepharose. The peptide RPRHFPQFSYSAS, corresponding to
17   residues 465-478 of PKBα, was synthesized on an Applied Biosystems
18   430A peptide synthesizer. cDNA encoding residues 46-400 of human
19   MAPKAP kinase-2 was expressed in E.coli as a glutathione
20   S-transferase fusion protein and activated with p38/RK MAP KINASE
21   by Mr A.Clifton (University of Dundee) as described previously
22   (Ben-Levy et al., 1995).
23
24   Monoclonal antibodies recognising the haemagglutonin (HA) epitope
25   sequence YPYDVPDYA, Protein G-Sepharose and histone H2B were
26   obtained from Boehringer (Lewes, UK). MAPKAP kinase-1 (Sutherland
27   et al., 1993) and p70 S6 kinases (Leighton et al., 1995) were
28   purified from rabbit skeletal muscle and rat liver respectively.
29
30   **Construction of expression vectors.** The pECE constructs encoding
31   the human HAPKBα and kinase-dead (K179A) HA-KD-PKBα have already
32   been described (Andjelkovic at al., 1996). The mutants at Ser-473
33   (HA-473A PKBα and HA-473D PKBα were created by PCR using a 5'
34   oligonucleotide encoding amino acids 406 – 414 and mutating 3'
35   oligonucleotide encoding amino acids 468 – 480, and the resulting
36   PCR products subcloned as *CelII-EcoRI* fragment into pECE.HA-PKBα.
37   The Thr-308 mutants (HA-308A PKBα and HA308D PKBα) were created by
38   the two-stage PCR technique (No et al., 1989) and subcloned as
39   *NotI-EcoRI* fragments into pECE.HA-PKB. The double mutant
40   HA-308D/473D PKB was made by subcloning the CelII-EcoRI fragment
41   encoding 473D into pECE.HA-308D PKBα. For construction of
42   cytomegalovirus-driven expression constructs, *BglII-XbaI* fragments
43   from the appropriate pECE constructs were subcloned into the same
44   restriction sites of the pCMV5 vector (Andersson et al., 1989).

1   All constructs were confirmed by restriction analysis and
2   sequencing and purified using Quiagen Plasmid Maxi Kit according
3   to the manufacturer's protocol. All oligonucleotide sequences are
4   available upon request.
5
6   ^{32}P-labelling of L6 myotubes and immunoprecipitation of PKBα. L6
7   cells were differentiated into myotubes on 10 cm diameter dishes
8   (Hundal et al., 1992). The myotubes were deprived of serum
9   overnight in DMEM, washed three times in phosphate free DMEM and
10   incubated for a further 1 h with 5 ml of this medium. The myotubes
11   were then washed twice with phosphate free DMEM and incubated for
12   4 h with carrier-free [^{32}P]orthophosphate (1 mCi/ml). Following
13   incubation in the presence or absence of 100 nM wortmannin for 10
14   min, the myotubes were stimulated for 5 min at 37°c in the
15   presence or absence of 100 nM insulin and placed on ice, The
16   medium was aspirated,the myotubes washed twice with ice-cold DMEM
17   buffer and then lysed with 1.0 ml of ice-cold Buffer A (50 mM
18   Tris/HCl pH 7.5,1 mM EDTA 1 mM EGTA, 1% (by vol) Triton X100, 1 mM
19   sodium orthopervanadate, 10 mM sodium glycerophosphate, 50 mM NaF,
20   5 mM sodium pyrophosphate, 1 μM Microcystin-LR, 0.27 M sucrose, 1
21   mM benzamidine, 0.2 mM phenylmethylsulphonyl fluoride, 10 μg/ml
22   leupeptin, and 0.1% (by vol) 2-mercaptoethanol). The lysates were
23   centrifuged at 4°C for 10 min at 13,000 x g and the supernatants
24   incubated for 30 min on a shaking platform with 50 μl of Protein
25   G-Sepharose coupled to 50 μg of preimmune sheep IgG. The
26   suspensions were centrifuged for 2 min at 13,000 x g and the
27   supernatants incubated for 60 min with 30 μl of Protein G--
28   Sepharose covalently coupled to 60 μg of PKBα antibody (Harlow and
29   Lane, 1988). The Protein G-Sepharose-antibody-PKBα complex was
30   washed eight times with 1.0 ml of Buffer A containing 0.5 M NaCl,
31   and twice with 50 mM Tris/HCl pH·7.5, 0.1 mM EGTA and 0.1% (by
32   vol) 2-mercaptoethanol (Buffer B).
33
34   Assay of immunoprecipitated PKBα and protein determinations. Three
35   aliquots of each immunoprecipitate (each comprising only 5% of the
36   total immunoprecipitated PKBα) were assayed for PKBα activity
37   towards the peptide GRPRTSSFAEG as described previously (Cross et
38   al., 1995). One unit of activity was that amount which catalysed
39   the phosphorylation of 1 nmol of substrate in 1 min. Protein
40   concentrations were determined by the method of Bradford, 1976.
41
42   Tryptic digestion of in vivo phosphorylated PKBα. The
43   immunoprecipitated PKBα was added to an equal volume of 2% (by

mass) SDS and 2 % (by vol) 2-mercaptoethanol, and incubated for 5
min at 100°C, After cooling to room temperature, 4-vinylpyridine
was added to a final concentration of 2 % (by vol) and the mixture
was incubated for 1h at 30°C on a shaking platform, followed by
electrophoresis on a 10% polyacrylamide gel. After
autoradiography, the 60 kDa band corresponding to rat PKBα was
excised and the gel piece homogenized in five vols of 25 mM
N-ethylmorpholine HCl, pH 7.7, containing 0.1% (by mass) SDS and 5
% (by vol) 2-mercaptoethanol. The suspension was incubated for 1 h
at 37°C on a shaking platform, then centrifuged for 1 min at
13,000 x g and the supernatant collected. The pellet was incubated
for a further 1h with five vols of the same buffer and centrifuged
for 1min at 13,000 xg. The two supernatants (containing 80-90% of
the ^{32}P-radioactivity) were combined, 0.2 vols of 100% (by mass)
trichloroacetic acid added, and the sample incubated for 1 h on
ice. The suspension was centrifuged for 10 min at 13,000 x g, the
supernatant discarded and the pellet washed five times with 0.2 ml
of water. The pellet was then incubated at 30°C with 0.3 ml of 50
mM Tris/HCl pH 8.0, 0.1% (by vol) Triton X100 containing 1μg of
alkylated trypsin. After 3 h another 1μg of trypsin was added and
the suspension left for a further 12 h. Guanidinium hydrochloride
(8 M) was added to bring the final concentration to 1.0 M in order
to precipitate any residual SDS and, after standing on ice for 10
min, the suspension was centrifuged for 5 min at 13, 000 x g. The
supernatant containing 90 % of the ^{32}P-radioactivity was
chromatographed on a Vydac C18 column as described in the legend
to Fig 2.

**Transfection of 293 cells and immunoprecipitation of HA-tagged
PKBα.** Human embryonic kidney 293 cells were cultured at 37°C in
an atmosphere of 5% CO2, on 10 cm diameter dishes in DMEM
containing 10 % foetal calf serum. Cells were split to a density
of 2 x 106 per 10 cm dish, and after 24 h at 37°C the medium was
aspirated and 10 ml of freshly prepared DMEM containing 10 %
foetal calf serum added. Cells were transfected by a modified
calcium phosphate method (Chen and Okayama, 1988) with 1ug/ml DNA
per plate. 10 μg of plasmid DNA in 0.45 ml of sterile water was
added to 50 μl of sterile 2.5 M CaCl2, and then 0.5 ml of a
sterile buffer composed of 50 mM N,N-bis[2-hydroxyethyl]-2-
aminoethanesulphonic acid/HCl pH 6.96, 0.28 M NaCl and 1.5 mM
Na2HPO4 was added. The resulting mixture was vortexed for 1 min,
allowed to stand at room temperature for 20 min, and then added
dropwise to a 10 cm dish of 293 cells). The cells were placed in

1   an atmosphere of 3% CO2, for 16 h at 37°C, then the medium was
2   aspirated, and replaced with fresh DMEM containing 10% foetal calf
3   serum. The cells were incubated for 12 h at 37°C in an atmosphere
4   of 5% CO2,, and then for 12 h in DMEM in the absence of serum.
5   Cells were preincubated for 10 min in the presence of 0.1% DMSO or
6   100 nM wortmannin in 0.1% DMSO and then stimulated for 10 min with
7   either 100 nM insulin or 50 ng/ml IGF-1 in the continued presence
8   of wortmannin. After washing twice with ice cold DMEM the cells
9   were lysed in 1.0 ml of icecold Buffer A, the lysate was
10   centrifuged at 4°C for 10 min at 13,000 x g and an aliquot of the
11   supernatant (10 μg protein) was incubated for 60 min on a shaking
12   platform with 5 μl of protein G-Sepharose coupled to 2 μg of HA
13   monoclonal antibody. The suspension was centrifuged for 1 min at
14   13,000 x g, the Protein G-Sepharose-antibody-HA-PKBα complex
15   washed twice with 1.0 ml of Buffer A containing 0.5 M NaCl, and
16   twice with Buffer B, and the immunoprecipitate assayed for PKBα
17   activity as described above.
18
19   ^{32}P-Labelling of 293 cells transfected with HA-PKBα. 293 cells
20   transfected with HA-PKBα DNA constructs. were washed with
21   phosphate free DMEM, incubated with [32p] orthophosphate (1
22   mCi/ml) as described for L6 myotubes, then stimulated with insulin
23   or IGF1 and lysed, and PKBα immunoprecipitated as described above.
24   The ^{32}P-labelled HA-PKBα immunoprecipitates were washed, alkylated
25   with 4-vinylpyridine, electrophoresed and digested with trypsin as
26   described above for the endogenous PKBα present in rat L6
27   myotubes.
28
29   Transfection of COS-1 cells and immunoprecipitation of HA-PKBα.
30   COS-1 cells were maintained in DMEM supplemented with 10% FCS at
31   370C in an atmosphere of 5% CO2,.· Cells at 70 – 80% confluency
32   were transfected by a DEAE-dextran method (Seed & Aruffo, 1987),
33   and 48 hours later serum-starved for 24 hours. Cells were lysed in
34   a buffer containing 50 mM Tris-HCl, pH 7.5,120 mM NaCl, 1% Nonidet
35   P-40, 25 mM NaF, 40 mM sodium-,β-glycerophosphate, 0.1 mM sodium
36   orthopervanadate, 1 mM EDTA, 1mM benzamidine, 1 mM
37   phenylmethylsulphonyl fluoride, and lysates centrifuged for 15 min
38   at 13,000 x g at 4°C. Supernatants were pre cleared once for 30
39   min at 4°C with 0.1 vols of 50% Sepharose 4B/25% Pansorbin
40   (Pharmacia and Calbiochem, respectively) and HA-PKBα
41   immunoprecipitated from 1 mg of extract using the 12CA5 antibody
42   coupled to Protein A Sepharose beads. Immunoprecipitates were
43   washed twice with lysis buffer containing 0.5 M NaCl and once with

1   lysis buffer.

2

3   **Immunoblotting and quantification of levels of PKBα expression.**
4   Cell extracts were resolved by 7.5% SDS-PAGE and transferred to
5   Immobilon membranes (Millipore). Filters were blocked for 30 min
6   in a blocking buffer containing 5% skimmed milk in 1x TBS, 1%
7   Triton X-100 and 0.5% Tween 20, followed by a 2h incubation with
8   the 12CA5 supernatant 1000-fold diluted in the same buffer. The
9   secondary antibody was alkaline conjugated anti-mouse Ig from goat
10   (Southern Biotechnology Associates, Inc), 1000-fold diluted in the
11   blocking buffer. Detection was performed using AP colour
12   development reagents from Bio-Rad according to the manufacturer's
13   instructions. Quantification of levels of PKBα expression was
14   achieved by chemiluminescence, using fluroisothiocyanante-labelled
15   antimouse IgG from goat as the secondary antibody and the Storm
16   840/860 and ImageQuant software from Molecular Dynamics.

17

18   All peptides used to assay PKBα, and TTYADFIASGRTGRRNAIHD (the
19   specific peptide inhibitor of cyclic AMP dependent protein kinase
20   - PKI) were synthesised on an Applied Biosystems 431A peptide
21   synthesizer. Their purity (> 95%) was established by HPLC and
22   electrospray mass spectrometry, and their concentrations were
23   determined by quantitative amino acid analysis.

24

25   **Preparation and assay of PKBα.** The construction of cytomegalovirus
26   vectors (pCMV5) of the human haemagglutonin epitope-tagged wild
27   type _(HA-PKBα) was described previously (Alessi et al., 1996).
28   293 cells grown on 10 cm dishes were transfected with a DNA
29   construct expressing HA-PKBα using a modified calcium phosphate
30   procedure (Alessi et al., 1996). The cells were deprived of serum
31   for 16h prior to lysis and, where indicated, were stimulated for
32   10 min in the presence of 50 ng/ml IGF-1 to activate PKBα. The
33   cells were lysed in 1.0 ml ice-cold Buffer A (50 mM Tris/HCl pH
34   7.5, 1 mM EDTA 1 mM EGTA, 1% (by vol) Triton X-100, 1 mM sodium
35   orthovanadate, 10 mM sodium β-glycerophosphate, 50 mM NaF, 5 mM
36   sodium pyrophosphate, 1 μM Microcystin-LR, 0.27 M sucrose, 1 mM
37   benzamidine, 0.2 mM phenylmethylsulphonyl fluoride, 10 μg/ml
38   leupeptin, and 0.1 % (by vol) 2-mercaptoethanol) the lysate
39   centrifuged at 4°C for 10 min at 13, 000 x g and the supernatant
40   obtained from one 10 cm dish of cells (2-3 mg protein) was
41   incubated for 60 min on a shaking platform with 20 μl of protein
42   G-Sepharose coupled to 10 μg of HA monoclonal antibody. The
43   suspension was centrifuged for 1 min at 13, 000 x g, the Protein

1   G-Sepharose-antibody-HA-PKBα complex washed twice with 1.0 ml of
2   Buffer A containing 0.5 M NaCl, and twice with Buffer B (50 mM
3   Tris/HCl pH 7.5, 0.1 mM EGTA, 0.01% (by vol) Brij-35 and 0.1% (by
4   vol) 2-mercaptoethanol). The PKBα immunoprecipitates were diluted
5   in Buffer B to an activity of 2.0 U/ml towards the Crosstide
6   peptide GRPRTSSFAEG and 0.1 ml aliquots snap frozen in liquid
7   nitrogen and stored at -80 oC. No significant loss of PKBα
8   activity occurred upon thawing the PKBα immunoprecipitates or
9   during storage at -80oC for up to 3 months . The standard
10   PKBα_assay (50 μl) contained: 50 mM Tris/HCl pH 7.5, 0.1 mM EGTA,
11   0.1% (by vol) 2-mercaptoethanol, 2.5 μM PKI, 0.2 U/ml PKBα,
12   Crosstide (30 μM), 10 mM magnesium acetate and 0.1 mM [γ^{32}P]ATP
13   (100-200 cpm/pmol). The assays were carried out for 15 min at
14   30oC, the assay tubes being agitated continuously to keep the
15   immunoprecipitate in suspension, then terminated and analysed as
16   described (Alessi et al., 1995). One unit of activity was that
17   amount of enzyme which catalysed the phosphorylation of 1 nmol of
18   Crosstide in 1 min. The phosphorylation of other peptides, histone
19   H2B and MBP were carried out in an identical manner. All the
20   Crosstide activity in HA-PKBα immunoprecipitates is catalysed by
21   PKBα (see Results) and the PKBα concentration in the
22   immunoprecipitates was estimated by densitometric scanning of
23   Coomassie blue-stained polyacrylamide gels, using bovine serum
24   albumin as a standard. Protein concentrations were determined by
25   the method of Bradford using bovine serum albumin as standard
26   (Bradford et al., 1976). Michaelis constants (Km) and Vmax values
27   were determined from double reciprocal plots of 1/V against 1/S,
28   where V is the initial rate of phosphorylation, and S is the
29   substrate concentration. The standard errors for all reported
30   kinetic constants were within $\leq \pm$ 20%, and the data is reported as
31   mean values for 3 independent determinations. Fig 16 shows the
32   results relative to those obtained for unstimulated PKBα.
33
34   **Tryptic digestion of histone 2B phosphorylated by PKBα.** Histone
35   H2B (30 μM) was phosphorylated with 0.2 U/ml HA-PKBα. After 60 min
36   0.2 vol of 100% (by mass) trichloroacetic acid was added, and the
37   sample incubated for 1 h on ice. The suspension was centrifuged
38   for 10 min at 13, 000 x g, the supernatant discarded and the
39   pellet washed five times with 0.2 ml of ice cold acetone. The
40   pellet was resuspended in 0.3 ml of 50 mM Tris/HCl pH 8.0, 0.1%
41   (by vol) reduced Triton-X100 containing 2 μg of alkylated trypsin
42   and, after incubation for 16 h at 30oC, the digest was centrifuged
43   for 5 min at 13, 000 x g. The supernatant, containing 95% of the

1 ^{32}P-radioactivity, was chromatographed on a Vydac C18 column

2 equilibrated with 0.1% (by vol) trifluoroacetic acid (TFA) in

3 water. With reference to the results shown in Fig 17, the columns

4 were developed with a linear acetonitrile gradient (diagonal line)

5 at a flow rate of 0.8 ml / min and fractions of 0.4 ml were

6 collected. (A) Tryptic peptide map of ^{32}P-labelled histone H2B,

7 70% of the radioactivity applied to the column was recovered from

8 the major ^{32}P-peptide eluting at 19.5% acetonitrile. (B) A portion

9 of the major ^{32}P-peptide (50 pmol) was analysed on an Applied

10 Biosystems 476A sequencer, and the phenylthiohydantoin (Pth) amino

11 acids identified after each cycle of Edman degradation are shown

12 using the single letter code for amino acids. A portion of the

13 major ^{32}P-peptide (1000 cpm) was then coupled covalently to a

14 Sequelon arylamine membrane and analysed on an Applied Biosystems

15 470A sequencer using the modified programme described in (Stokoe

16 et al., 1992). ^{32}P radioactivity was measured after each cycle of

17 Edman degradation.

18

19

Table 7.1

Molecular basis for the substrate specificity of PKBα

The phosphorylated residue is shown in boldface type, the altered residue is underlined. V(100 μM) is the relative rate of phosphorylation at 0.1 mM peptide relative to peptide 1. ND, not determined. *An alanine residue was added to the C-terminal of the peptide RPRTSSP, since we have experienced difficulty in synthesing peptides terminating in proline.

A	Peptides	Km (μM)	Vmax (U/mg)	V(0.1 mM)
1.	GRPRTSSFAEG	4	250	100
2.	RPRTSSFA	8	305	109
3.	GRPRTSSF	8	385	129
4.	RPRTSSF	5	260	105
5.	RPRTSTF	30	243	78
6.	RPRTSAF	–	0	
7.	PRTSSF	–	0	
8.	RPRTSS	>500	ND	2
9.	KPRTSSF	>500	ND	4
10.	RPKTSSF	>500	ND	2

B				
1.	RPRTSSF	5	260	105
2.	RPRTSSL	8	278	104
3.	RPRTSSV	21	300	102
4.	RPRTSSA	250	265	30
5.	RPRTSSK	80	308	67
6.	RPRTSSE	>500	ND	9
7.	RPRTSSPA*	–	0	

C				
1.	RPRTSSF	5	260	105
2.	RPRASSF	12	230	89
3.	RPRVSSF	25	273	77
4.	RPRGSSF	60	163	37
5.	RPRNSSF	>500	ND	21
6.	RPRTASF	20	213	83
7.	RPRTGSF	25	233	77
8.	RPRTVSF	30	365	89
9.	RPRTNSF	30	300	81
10.	RPRAASF	25	215	77
11.	RPRGGSF	105	345	55
12.	RPRGASF	105	160	37
13.	RPRAGSF	49	114	70

Table 7.2 Comparison of the substrate specificities of PKBα, MAPKAP kinase-1, and p70S6 kinase. Peptides 1 and 2 are very good substrates for MAPKAP kinase-1 and p70 S6 kinase, and peptide 3 is a relatively specific substrate for p70 S6 kinase [16]. *Data reported previously [16]; ND, not determined.

A

Peptide	Protein kinase Bα		MAPKAP kinase-1		p70 S6 kinase	
	K_m (mM)	V_{max} (U/mg)	K_m (mM)	V_{max} (U/mg)	K_m (mM)	V_{max} (U/mg)
1. KKKNRTLSVA	185	270	0.2*	1550*	3.3*	890*
2. KKRNRTLSVA	80	300	0.7*	1800*	1.5*	1520*
3. KKRNKTLSVA	>500	ND	17*	840*	34*	760*
4. KKRNRTLTV	388	330	40*	270*	4.8*	1470*

B

Peptide	K_m (mM)	V_{max} (U/mg)	K_m (mM)	V_{max} (U/mg)	K_m (mM)	V_{max} (U/mg)
1. GRPRTSSFAEG	4	250	2	790	3	1270
2. RPRTSSF	5	260	12	840	125	705
3. RPRTSTF	30	240	>500	ND	211	590
4. RPRAASF	25	215	20	1020	>500	ND
5. RPRAATF	25	230	>500	ND	>500	ND

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